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HALLYSSON DOUGLAS ANDRADE DE ARAÚJO

TOXICIDADE AGUDA E AVALIAÇÃO DO SAL DE POTÁSSIO DO ÁCIDO ÚSNICO SOBRE ESTÁGIOS EVOLUTIVOS DO *Schistosoma mansoni* E DO SEU HOSPEDEIRO INTERMEDIÁRIO

Recife
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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco - UFPE.
Área de concentração em Bioquímica e Fisiologia.

Orientadora: Profª. Drª. Vera Lúcia de Menezes Lima

Coorientador: Profº. Dr. André de Lima Aires

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Profª. Drª. Luana Cassandra Breitenbach Barroso Coelho
(Membro Interno - Presidente)

Profª. Drª. Glória Isolina Boente Pinto Duarte
(Membro Interno)

Prof. Dr. Naftale Katz
(Membro Externo)

Profª. Drª. Bianka Santana dos Santos
(Membro Externo)

Profº. Dr. Caíque Silveira Martins da Fonseca
(Membro Externo)

Dedico esta tese a minha esposa Silvânia Araújo e aos meus filhos Lavínya Araújo e Samuel Araújo. A vida acadêmica é gratificante, porém me fez estar ausente de bons momentos com vocês!

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``Posso todas as coisas em **Cristo** que me fortalece``

(FILIPENSES. c. 4 v. 13)

RESUMO

A existência apenas do praziquantel e do niclosamida para o tratamento da esquistossomose e controle dos hospedeiros intermediários do *Schistosoma mansoni* incentiva a busca por novos agentes esquistossomicidas e moluscicidas. O presente estudo avaliou os efeitos do sal de potássio derivado do ácido úsnico (SP-AU) sobre os estágios evolutivas da *Biomphalaria glabrata* (blástula (E1), gástrula (E2), trocofora (E3), véliger (E4) e *hippo stage* (E5)) e *S. mansoni* linhagem BH, (cercárias (S1), esquistossômulos (S2), vermes jovens (S3) e vermes adultos (S4)), também foi avaliada a toxicidade aguda do SP-AU em modelo murino. O ácido úsnico foi extraído e purificado do líquen *Cladonia substellata* e modificado em SP-AU. A molécula do SP-AU foi confirmada por RMN de ^{1}H e ^{13}C, IV e análise elementar. Em relação a embriotoxicidade as concentrações 3 e 5 µg/mL do SP-AU provocaram 26 e 49% de inviabilidade no estágio E1, enquanto 2 e 3 µg/mL obtiveram percentuais semelhantes em E2. Os embriões em E3, E4 e E5 também foram afetados, em 2,5 µg/mL foram necessários para causar 27, 26 e 46% de malformações ou morte, respectivamente. Os 100% de letalidade dos embriões E1 a E5 foram observados nas concentrações 6,0, 4,0, 4,5, 4,5 e 4,0 µg/mL, respectivamente. Enquanto, para S1 após 2 h de exposição em 2,41 µg/mL do SP-AU 90% estavam mortas. A exposição dos vermes S2, S3 e S4 ao SP-AU foi observado uma motilidade reduzida, inviabilidade celular, diversificados danos tegumentares e letalidade após 24 h de exposição em 12,5, 200 e 100 µM respectivamente. Os S4 expostos às concentrações subletais 50, 25 e 12,5 µM, nos intervalos de 24 a 120 h não evidênciaram presença de ovos e as mortes foram dose tempo resposta. Em relação a toxicidade aguda o SP-AU em 500 mg/kg não ocasionou alterações homeostáticas. Enquanto, o consumo de alimentos e água diminuiu com os tratamentos de 1000 e 2000 mg/kg, seguidos de leucócitos e transaminases hepáticas aumentados e diminuição do colesterol e triglicérides para ambos tratamentos. Alterações histológicas do fígado e rim incluindo mortes foram detectadas apenas em 2000 mg/kg. Alterações morfológicas e mortes dos embriões da *B. glabrata* e vermes de *S. mansoni* reforça que o SP-AU é um promissor agente moluscicida e esquistossomicida, atuando assim no controle da esquistossomose mansônica, além disto, sendo considerado levemente tóxico em modelo murino quando administrado por via oral de acordo com os parâmetros internacionais.

Palavras-Chave: líquen; ácido úsnico; droga solúvel; atividade embriotóxica; *Biomphlaria glabrata*; atividade esquistossomicida; *Schistosoma mansoni*.

ABSTRACT

The existence of only praziquantel and niclosamide for the treatment and control of schistosomiasis and the intermediate host of *Schistosoma mansoni* encourages the search for new schistosomicidal and molluscicidal agents. In the present study the acute *in vivo* toxicity and the *in vitro* effect of potassium salt derived from unic acid (PS-UA) on evolutionary stages of *S. mansoni* BH strain (cercariae (S1), schistosomules (S2), young worms (S3) and adult (S4)) and and embryos of *Biomphalaria glabrata* (blastula (E1), gastrula (E2), trocophora (E3), veliger (E4) and *hippo stage* (E5)) were evaluated. To obtain the PS-UA, the usnic acid was extracted and purified from lichen *Cladonia substellata* and modified in PS-UA. The molecule was confirmed by ^{1}H and ^{13}C NMR, IV and elemental analysis. Toxicity results showed that PS-UA did not show deaths below 2000 mg/kg and was considered safe or slightly toxic when administered orally. Food and water consumption decreased with 1000 and 2000 mg/kg treatments, followed by increased leukocytes and decreased cholesterol and triglycerides, there was an increase in liver transaminases for both treatments. Histological changes in liver and kidney were detected only at 2000 mg/kg. In relation to embryotoxicity, concentrations 3 and 5 μg/mL of PS-UA caused 26 and 49% of unviable in stage E1, while 2 and 3 μg/mL obtained similar percentages in E2. Embryos at E3, E4 and E5 were also affected, only 2.5 μg/mL were required to cause 27, 26 and 46% of malformations or death, respectively. 100% lethality were observed at the concentrations 6.0, 4.0, 4.5, 4.5 and 4.0 μg/mL, respectively. While, in vitro assays the exposure of PS-UA showed reduced motility and caused the death of the parasite, whose effects were time and concentration dependent. S1 presented LC_{90} of 2.41 μg/mL after 2h exposure in aqueous medium. S2, S3 and S4 did not present lethality corresponding to the evolutionary phase of *S. mansoni* with LC_{100} (24 h) in respective 12.5, 200 and 100 μM. S4 in all ranges from 24 to 120 h sublethal concentrations (50, 25 and 12.5 μM) there was no oviposition of female worms of *S. mansoni*. The PS-UA caused severe, extensive and diverse tegumentary damage in the evolutionary phases of *S. mansoni* as toxic and teratogenic effect in the embryonic stages of *B. glabrata*, reinforcing that the PS-UA is a promising drug candidate for an effective molluscicidal agent, thus acting in the control of schistosomiasis mansoni.

Keywords: lichen; usnic acid; soluble drug; embryotoxic activity; *Biomphlaria glabrata*; schistosomicidal activity; *Schistosoma mansoni*.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

Adenonosina Trifosfato (ATP)

Agência dos Estados Unidos para o Desenvolvimento Internacional (USAID)

Biomphalaria glabrata (*B. glabrata*)

Biomphalaria straminea (*B. straminea*)

Biomphalaria tenagophila (*B. tenagophila*)

Concentração inibitória (CI)

Concentração letal (CL)

Dose letal (DL)

Doenças tropicais negligênciadas (DTNs)

Hospital das Clínicas da Universidade Federal de Pernambuco (HC-UFPE)

Interleucina (IL)

Organização para a Cooperação e Desenvolvimento Económico (OECD)

Oxamniquini (OXA)

Praziquantel (PZQ)

Programa de controle da esquistossomose (PCE)

Schistosoma guineensis (*S. guineensis*)

Schistosoma haematobium (*S. haematobium*)

Schistosoma intercalatum (*S. intercalatum*)

Schistosoma japonicum (*S. japonicum*)

Schistosoma mansoni (*S. mansoni*)

Schistosoma mekongi (*S. mekongi*)

Ultravioleta (UV)

SUMÁRIO

1 INTRODUÇÃO

A esquistossomose é uma infecção crônica, potencialmente fatal com manifestações que acometem o sistema hepatoesplênico e trato gastrointestinal envolvendo múltiplos mecanismos diretamente relacionados aos antígenos de suas diferentes fases evolutivas em especial as reações inflamatórias granulomatosas frentes aos antígenos dos ovos (McMANUS et al., 2018; SCHWARTZ; FALLON, 2018). A infecção é endêmica em 78 países e territórios, em regiões tropicais e subtropicais, onde mais de 220 milhões de pessoas são portadoras, 800 milhões vivem sob risco iminente de infecção e aproximadamente 200 mil morrem todos os anos (STEINMANN et al., 2006; UTZINGER et al., 2011; WHO, 2020).

No Brasil a esquistossomose encontra-se presente em todas as regiões brasileiras com estimativa de 1,5 milhões de infectados pelo *S. mansoni*, única espécie encontrada no continente Americano (KATZ, 2008; MARTINS-MELO et al., 2014). Pernambuco é o terceiro estado com maior incidência, e o primeiro em óbitos, cuja proporcionalidade é três vezes maior que a média nacional (MARTINS-MELO et al., 2014; PERNAMBUCO, 2014). A transmissão ocorre em ambientes aquáticos contaminados com cercarias, fase evolutiva infectante para o hospedeiro definitivo, que são liberadas por caramujos do gênero *Biomphalaria* spp. hospedeiros intermediários. No Brasil as principais espécies naturalmente infectadas pelos *S. mansoni* são: *B. glabrata*, *B. tenagophila* e *B. straminea* (SCHOLTE et al., 2012). Essas espécies vivem preferencialmente nas margens das coleções de águas doce rasas, de pouca correnteza ou águas paradas tais como: lagoas, lagos, poços, remansos de rios, riachos e canais de irrigação (KAWANO, et al., 2008; BRASIL, 2014). Os moluscos quando alcançam a maturidade sexual repovoam rapidamente os criadouros, por serem hermafroditas, reproduzem cerva de 10 milhões de descendentes em menos de 3 meses (BRASIL, 2014).

Atualmente, o Praziquantel (PZQ) é a única droga recomendada pela Organização Mundial da Saúde (OMS) para reduzir a prevalência e incidência da esquistossomose em todo o mundo, desde que, não existe vacina segura e eficaz contra o *Schistosoma* spp. (CIOLI et al., 2014; DINIZ et al., 2014). PZQ, desenvolvido na década de 70, apesar de seguro e eficaz, contra vermes adultos, não atua sobre as reações inflamatórias granulomatosas, não é eficaz contra os estágios evolutivos do *S. mansoni* (esquistossômulos de pele, esquistossômulos pulmonar e vermes jovens), além de não previne a reinfecção (SABAH et al., 1986; DINIZ et al., 2014; OLIVEIRA et al., 2014). Ademais, é preocupante a disponibilidade de apenas uma droga para tratar uma doença em expansão e estudos relatam o desenvolvimento de cepas de *Schistosoma* spp. com baixa sensibilidade e/ou resistência ao PZQ (ISMAIL et al., 1999;

MELMAN et al., 2009). Com relação ao controle populacional dos hospedeiros intermediários, o Programa de Controle da Esquistossomose orienta a aplicação do moluscicida niclosamida em áreas endêmicas. Entretanto, a o uso de niclosamida tem gerado preocupação em relação a vários fatores dentre eles, altamente tóxico para o ecossistema aquático e custo elevado do produto aos países endêmicos (KING; BERTSCH, 2015). Esse cenário tem motivado nosso grupo a contribuir com a pesquisa e o desenvolvimento de novos fármacos, naturais ou derivados, com potencial esquistossomicida e moluscicida.

Na literatura diversos estudos exploram o potencial esquistossomicida e moluscicida de origem natural, semi-natural e sintéticos (AIRES et al., 2014; ALBUQUERQUE et al., 2014; OLIVEIRA et al., 2014; SANTOS et al., 2014; NEVES; ANDRADE; CRAVO, 2015; SILVA et al., 2018; 2019). Os produtos naturais são importantes fontes para a pesquisa farmacológica e o desenvolvimento de medicamentos, não somente quando seus constituintes são usados diretamente como agentes terapêuticos, mas também como matérias-primas para a derivados semi-naturais ou síntese (BRASIL, 2006). Neste sentido os líquens, são seres simbióticos muito encontrados em regiões tropicais e subtropicais (AHTI, 2000), apresentam em sua estrutura fungos (micobionte, heterotrófico) e algas verdes ou cianobacteria (fotobionte, autotrófico), que produzem metabólitos secundários naturais, sendo quantificados em mais de 1.000 compostos, onde uma média de 80% dessas moléculas são exclusivos dessa simbiose (YOUSUF et al., 2014). O ácido úsnico é um metabólito secundário de líquen presentes nos gêneros *Cladonia*, *Usnea*, *Lecanora*, *Ramalina*, *Parmelia* e *Evernia* e desempenha importante papel biológico, conferindo aos liquens proteção contra microrganismo invasores, raios UV e ressecamento do talo (COCCHIETTO et al., 2002; INGÓLFSDÓTTIR et al., 2002). Esta molécula tem sido alvo recente de estudos por apresentar promissoras atividades biológicas tás como: cicatrizante, antiviral, anti-flamatória, antioxidante, antimicrobiana e antiparasitárias (WHITE et al., 2014).

Entretanto, o ácido úsnico tem a desvantagem por apresentar baixa solubilidade aquosa e alta toxicidade em modelos *in vitro* e *in vivo* devido às suas características hidrofóbicas relacionadas às suas propriedades físico-químicas (COCCHIETTO et al., 2002; INGÓLFSDÓTTIR et al., 2002; WHITE et al., 2014; SIGMA-ALDRICH, 2018). No entanto, através de uma reação ácido-base (base hidróxido de potássio) com o ácido úsnico obtém o sal de potássio do ácido úsnico e poucos estudos de suas propriedades biológicas e farmacológicas, tem sido repotada(s), excetos a atividade moluscicida frente ao *B. glabrata* adultos oriundos de Nazaré da Mata-PE (MARTINS et al., 2014) e inibições das metástases hepáticas e do câncer colorretal em modelo murino (YANG et al., 2018). Apesar de todos os

avanços biotecnológicos (biologia molecular, genômica, química médica e analítica, modelagem matemática e computacional) para descobrir os mecanismos de ação de canditados a fármacos por exemplo, aos agentes esquistossomicidas para o tratamento da esquistossomose sobre todos os estágios evolutivos do *Schistosoma* spp. precisam também apresentar limites toleráveis de toxicidade (CAMPELO et al., 2018; MAFUD et al., 2018).

Diante do exposto, objetivamos explorar o potencial esquistossomicida do sal de potássio do ácido úsnico sobre diferentes fases evolutivas do *S. mansoni* e do seu hospedeiross intermediário (embriões de *B. glabrata*), avaliando também a toxicidade aguda em modelo murino do sal de potássio do ácido úsnico.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Susceptibilidade das fases evolutivas do *Schistosoma mansoni* e do seu hospedeiro intermediário frente ao sal de potássio do ácido úsnico e sua toxicidade aguda em modelo murino.

1.1.2 Objetivos específicos

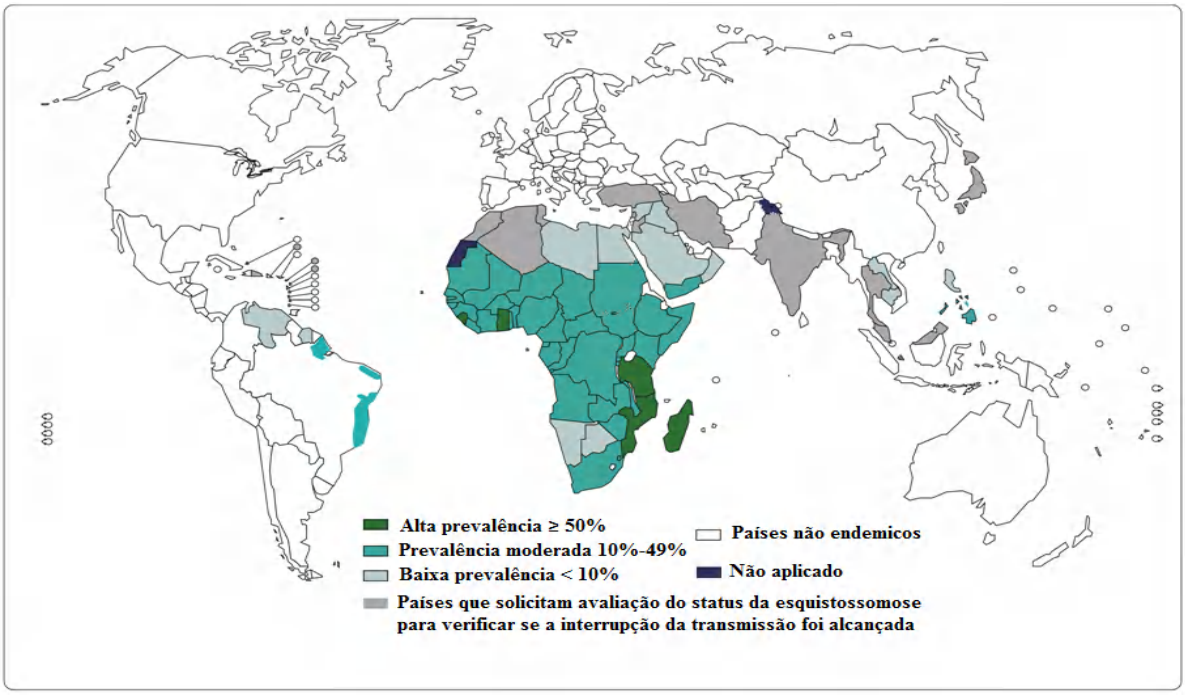
- Coleta do líquen *Cladonia substellata*, purificação do ácido úsnico e obtenção do sal de potássio do ácido úsnico (SP-AU) e sua caracterização;
- Avaliar a atividade embriotóxica do SP-AU sobre os estágios embrionários (blástula, gástrula, trocófora, véliger e *hippo stage*) da *B. glabrata*;
- Avaliação *In Vitro* das fases evolutivas (cercárias, esquistossômulos, vermes jovens e adultos) do *S. mansoni* frente ao SP-AU;
- Avaliar a citotoxicidade do SP-AU sobre células mononucleares de sangue periférico (PBMCs);
- Avaliar a toxicidade aguda em modelo murino do SP-AU.

2 REVISÃO BIBLIOGRÁFICA

2.1 EPIDEMIOLOGIA DA ESQUISTOSSOMOSE

Mundialmente, a esquistossomose encontra-se presente em 78 países e territórios das regiões tropicais e subtropicais abrangendo a América do Sul e Caribe, Ásia, África e Leste do Mediterrâneo, sendo também recentemente reportada em países europeus (Figura 1). A doença atinge cerca de 220 milhões de pessoas nessas regiões, com estimativa de que 800 milhões vivam sob risco iminente de infecção e 200 mil morrem anualmente (STEINMANN et al., 2006; UTZINGER et al., 2011, WHO, 2020).

Figura 1 - Distribuição da Esquistossomose no Mundo



Fonte: Adaptado de Lu et al. (2018).

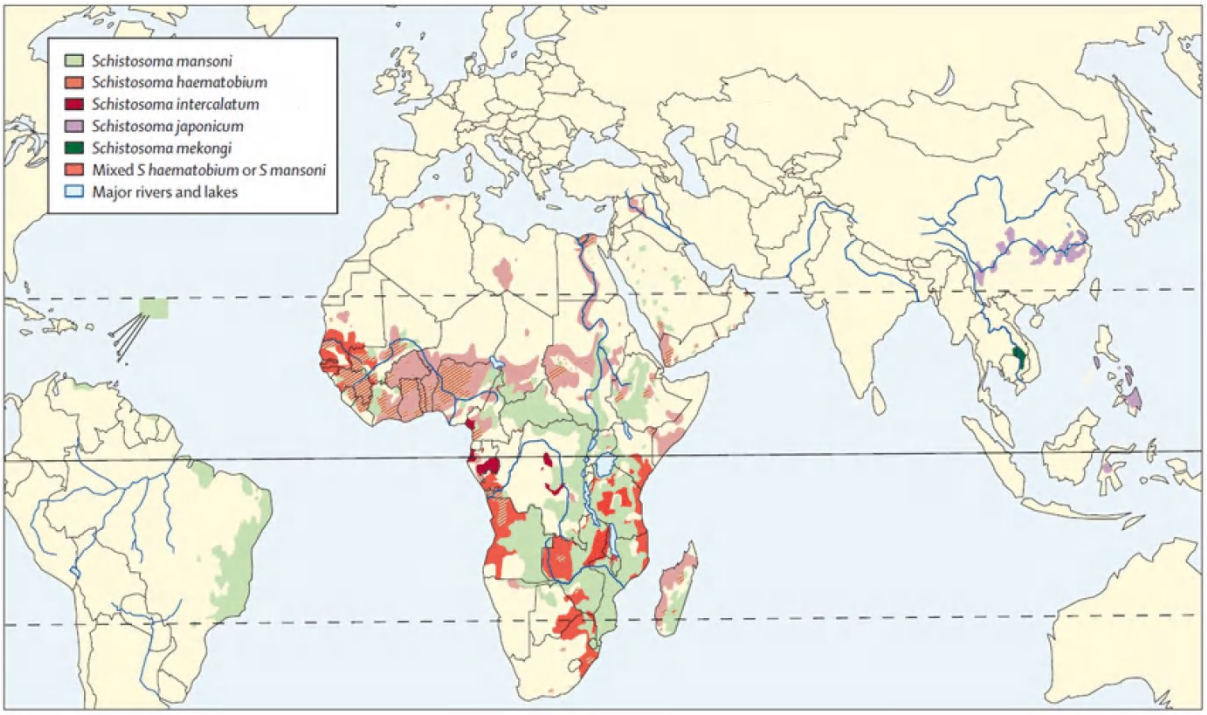
Os agentes etiológicos pertencem ao gênero *Schistosoma* spp. são quantificados em 7 espécies *Schistosoma guineensis, S. haematobium, S. intercalatum, S. japonicum, S. mansoni*, *S. mekongi* e *S. malayensis*, encontrados dispersos em diferentes continentes e em alguns países apresentam de 2 a 3 agentes etiológicos diferentes em um mesmo território (Figura 2) (ELBAZ; ESMAT, 2013; COLLEY et al., 2014).

Cerca de 72% dos indivíduos com esquistossomose vivem em países africanos (WHO, 2016, 2019). A prevalência de esquistossomose ainda é alta na África Subsaariana, na qual é

formada por 48 países. Em 2008, 17,5 milhões de pessoas foram tratadas globalmente para esquistossomose, sendo deste total 11,7 milhões presentes apenas na África Subsaariana.

Atualmente, aproximadamente 120 milhões de indivíduos na África subsaariana apresentam-se infectados com a esquistossomose, dentre eles a Etiópia revelou altíssima prevalência da doença, com cerca 75% de positividade na população do país, sendo também apresentadas formas graves da doença como hepatomegalia, esplenomegalia e fibrose periportal nos Etíopes (ADENOWO et al., 2015). Também foram relatados importantes dados epidemiológicos alarmantes, com um total de mais de 60 milhões de pessoas infectadas na Nigéria, 18 milhões na República Democrática do Congo, 13 milhões em Moçambique, 11 milhões no Quênia, enquanto República Unida da Tanzânia apresenta mais de 10 milhões de parasitados para *Schistosoma* spp. (ADENOWO et al., 2015).

Figura 2 - Distribuição das espécies de *Schistosoma* spp. no Mundo



Fonte: Colley et al. (2014).

Em 2000, a colaboração entre o Ministério da Saúde do Egito e a Agência dos Estados Unidos para o Desenvolvimento Internacional (USAID), no "Projeto de Pesquisa da Esquistossomose", alcançou seus objetivos propostos através de uma amostra aleatória de moradores de comunidades rurais de nove províncias de várias partes do Egito (Alto e Baixo

Egito). A pesquisa revelou que a prevalência da infecção por *S. haematobium* no Alto Egito variou de 4,8% a 13,7% com a prevalência de pico de infecção estratificada por idade na faixa etária de 10 a 14 anos. Enquanto, no Baixo Egito na cidade de Ismailia, uma cidade do Canal de Suez, a maior taxa de infecção foi de 1,8% para o *S. haematobium*. Enquanto, a infecção ocasionada pelo *S. mansoni* foi considera rara no Alto Egito, com exceção apenas na cidade de Fayoum com uma prevalência de 4,3%, enquanto no Baixo Egito a prevalência variou de 17,5% a 42,9%, com maior pico de prevalência da infecção na faixa etária de 15 aos 19 anos (EL-KHOBY et al., 2000).

No entanto, na área de Abis (próximo de Alexandria), que é supostamente uma região de baixa prevalência e intensidade de *S. mansoni*, 15,2% das crianças de 11 anos de idade foram infectadas com *S. mansoni* com carga parasitária de 480 ovos/g fezes (ALLAM et al., 2009). Da mesma forma, em um estudo da comunidade de artesãos em 2014 no Lago Manzala, no norte do delta do Nilo, a prevalência de infecção por *S. mansoni* foi tão alta quanto 26,6% com uma intensidade de 42,7 ± 7 ovos/g de fezes (TAMAN et al., 2014).

Em relação à presença da esquistossomose na Europa, recentemente foram identificados surtos da esquistossomose urogenital nos países da França, Alemanha e Itália, acreditando-se que a infecção tenha se iniciado na região Sul da Europa, na cidade de Córsega - França, em um rio ao norte de Porto-Vecchio, um destino turístico popular. Provavelmente, a chegada da esquistossomose nestes países esteja relacionada com a migração de pessoas infectadas de regiões endêmicas da África, para a Europa ou associada com o turismo, após os turistas europeus retornarem de áreas endêmicas na África após terem sidos contaminados ao entrarem em contato com águas com cercarias de *S. haematobium* já que o parasita é uma espécie encontrada apenas nos países do continente africano (BOISSIER et al., 2015).

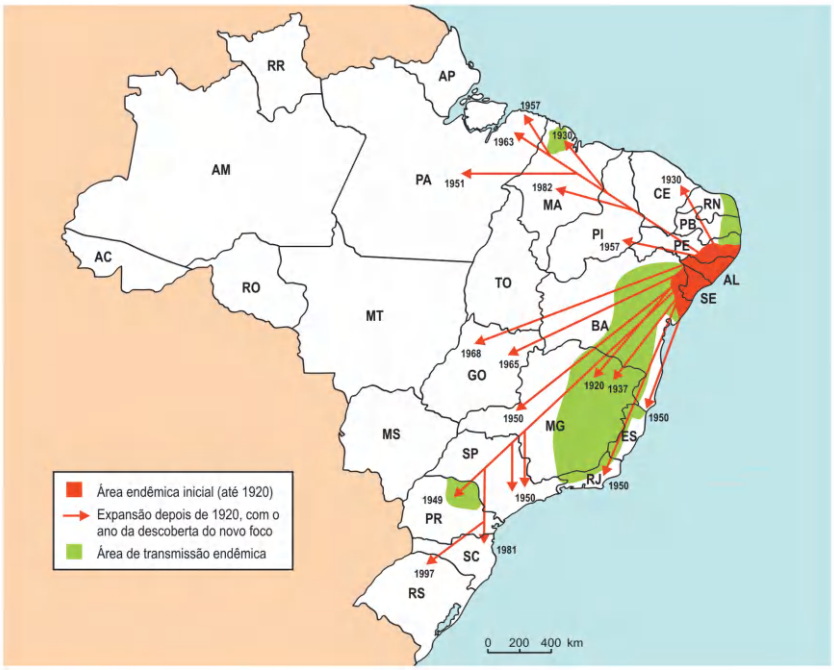
O Brasil é o país da América Latina que apresenta os maiores índices de pessoas infectadas por esquistossomose. Acredita-se que a doença adentrou no país no período do Brasil colônia, através dos escravos que se encontravam parasitados oriundos da costa ocidental do continente africano, que foram trazidos pelo sistema escravocrata para trabalharem nas lavouras da monocultura da cana-de-açúcar (NOYA et al., 2015).

Esses escravos desembarcaram e se instalaram inicialmente na Região do Nordeste do Brasil, precisamente nos Portos marítimos dos Estados da Bahia e Pernambuco. Alguns fatos históricos e socioeconômicos, juntamente com os fatores de ordem bioecológicas (para que se completasse o ciclo evolutivo do parasita), foram responsáveis pela expressão da endemia em ambos os estados (NOYA et al., 2015). Tradicionalmente, o cultivo da cana-de-açúcar pelas capitanias de Pernambuco e da Bahia foi desenvolvido nos vales úmidos (irrigados por muitos

rios) e colinas suaves, originariamente ocupada por vegetação típica de floresta (Mata Atlântica) que se estendia perpendicularmente ao litoral (ANDRADE, 2001).

No século XVII, surgem movimento migratório orientado para o interior de diferentes estados, destinado a implementar a criação de gado, com a finalidade de abastecer o mercado aberto com a colonização do litoral nordestino, decorrente da exploração de cana-de-açúcar. É possível que o translado de mão-de-obra escrava, fixada inicialmente na orla litorânea, para o interior, tenha iniciado o deslocamento da área endêmica de esquistossomose para regiões onde, até hoje, são elevados os índices de infecção autóctone, como alguns municípios dos estados da Paraíba, Bahia, Sergipe, Alagoas e Pernambuco (BRASIL, 2014). No século XVIII, criaram-se condições para que a esquistossomose viesse a expandir atingindo áreas no interior de diversos outros Estados (Figura 3), por exemplo, Minas Gerais, atraídos pela descoberta de jazidas auríferas e de outros minerais preciosos cuja exploração, no correr do século, constituiram a principal atividade econômica do país (BRASIL, 2014).

Figura 3 - Representação da expansão da esquistossomose mansônica no Brasil.

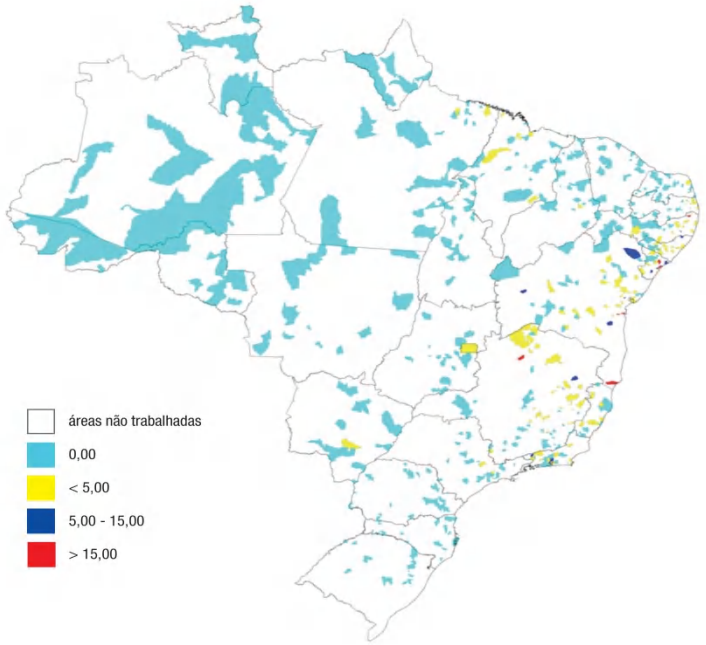


Fonte: Brasil, (2014).

Atualmente, no Brasil, estima-se que pelo menos 1,5 milhões de brasileiros encontram-se infectadas pelo *S. mansoni* (Figura 4) (NOYA et al. 2015; KATZ, 2018) sendo que 400 a 500 mortes são relatadas anualmente (MARTINS-MELO et al., 2014) e outras 25 milhões de pessoas vivem em áreas endêmicas com risco de contrair a doença distribuídas em

todas as regiões brasileiras (NOYA et al., 2015; PAN AMERICAN HEALTH ORGANIZATION, 2020). No Brasil é observado diferentes faixas de prevalência nos 14 estados, mais intensamente distribuída numa faixa de terras contínuas e contíguas ao longo de quase toda a costa litorânea brasileira, incluindo principalmente as zonas quentes e úmidas seguindo o trajeto de importantes bacias hidrográficas desses estados, entre os estados brasileiros destacam-se; Maranhão, Paraíba, Rio Grande do Norte, Pará, Piauí, Rio de Janeiro, São Paulo e Paraná, sendo as áreas mais endêmicas/prevalentes encontradas nas regiões Nordeste e Sudeste representados pelos Estados: Pernambuco, Bahia, Alagoas, Sergipe, Norte de Minas Gerais e Espírito Santo (BRASIL, 2014; SCHOLTE et al., 2014; KATZ et al., 2018).

Figura 4. Distribuição da esquistossomose mansônica de acordo com faixa de prevalência, por município. Brasil.



Fonte: Katz, (2018).

Ainda no Brasil, de acordo com os dados oficiais do manual de vigilância da esquistossomose mansoni do Ministério da Saúde, entre 2008 a 2012 ocorreram 1.722 internações por 100 mil habitantes e 2.503 óbitos por esquistossomose na mesma temporalidade (BRASIL, 2014), esta situação classifica a doença como sendo a 100ª causa de

morte no Brasil, e que as mortes por esquistossomose estimadas no Brasil representaram 3,6% do total mundial (GLOBAL HEALTH DATA EXCHANGE, 2018).

O Brasil está apresentando melhoria considerável na gravidade da doença e uma diminuição gradativa nos números de pessoas infectadas, internações hospitalares e óbitos relacionados às hemorragias digestivas nos casos hepatoesplênicos da esquistossomose, em comparação a décadas anteriores, com o uso rotineiro do tratamento específico com quimioprofilaxia menos tóxica em massa, quando necessário, atuação dos programas de educação em saúde continuada nas escolas e unidades de saúde e aplicações de moluscicida nos recursos hídricos (apenas nos locais de alta prevalência), onde se encontra(m) o(s) hospedeiro(s) intermediário(s) da doença (SILVA; LEAL; DOMINGUES, 2013; MARTINS-MELO 2015; 2016; WHO, 2017; FACCHINI et al., 2018).

Embora este cenário mostre-se positivo, algo ainda precisa ser revisto pelas autoridades públicas que executam ou são responsáveis pela fiscalização dos programas de controle da esquistossomose (PCE), para que estes mesmos números venham continuar a decrescer. Foi divulgado pelo Ministério da Saúde entre os anos de 2008 a 2012 os números de pessoas infectadas e tratada para a esquistossomose e o cenário observado foi bastante preocupante, sendo observados que aproximadamente 50 mil brasileiros com diagnóstico positivo para esquistossomose não fizeram uso da medicação nos respectivos anos (BRASIL, 2014). Este reflexo foge do foco da obrigatoriedade que os PCE devem fazer com a busca ativa aos doentes, apresentando assim um déficit muito perigoso, principalmente para o país que é signatário da Resolução WHA65-21, da Organização Mundial da Saúde que propõe a eliminação da transmissão da esquistossomose (WHO, 2012), além de que esta situação (ausência da busca ativa) está permitindo que ocorra uma expansão da esquistossomose mânsonica através do fluxo das pessoas parasitadas (BARBOSA et al., 2015).

Ainda sobre a expansão da doença para áreas antes nunca descritas, como por exemplo para comunidades litorâneas, devido às constantes migrações da população rural para as periferias dos centros urbanos e turísticos, propiciando devastação ambiental e uso e ocupação desordenada do solo por imigrantes que buscam principalmente oportunidades financeiras, estes antecedentes tem contribuido para a expansão da doença, tendo sido já reportado por Gomes et al. (2014) e Barbosa et al. (2015) através de estudos epidemiológicos de campo.

Nessas localidades litorâneas, a transmissão é sazonal, ocorrendo em períodos chuvosos, quando as lagoas em áreas peridomiciliares que formam os focos de infecção estouram e os caramujos infectados são transportados passivamente pela chuva para as ruas e

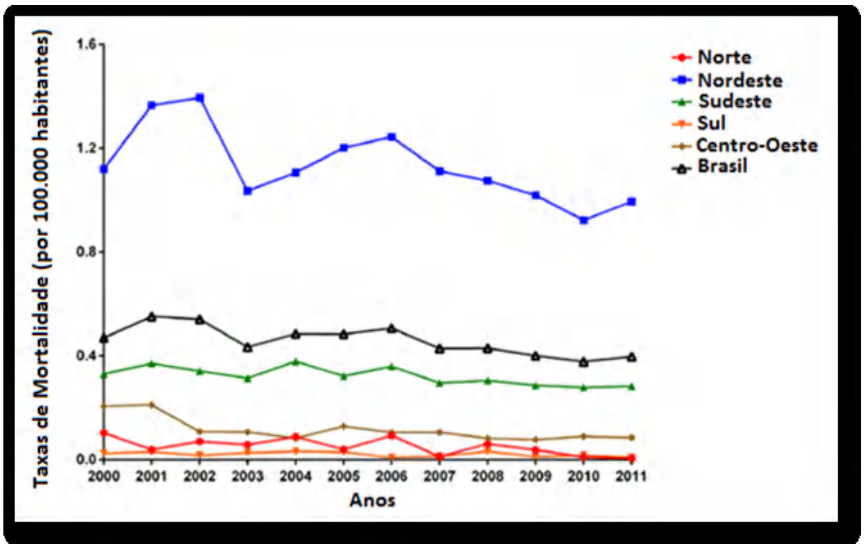
quintais, expondo residentes e turistas a uma infecção inicial. Este tipo de transmissão foi registrado em um surto de esquistossomose aguda, que ocorreu na praia de Porto de Galinhas, litoral Sul de Pernambuco, em 2000, com 662 casos positivos para *S. mansoni* (BARBOSA et al., 2001). Em 2010, uma nova pesquisa realizada naquela localidade diagnosticou 425 novos casos de esquistossomose. A falta de saneamento e poluição do meio ambiente permitiram que a doença se tornasse endêmica nessa área (BARBOSA et al., 2011; GOMES et al., 2014). Em 2014, o mesmo processo foi percebido e relatado pela primeira vez na comunidade litorânea de Serrambi, Pernambuco, onde um inquérito parasitológico com 1.414 indivíduos (54,9% da população) diagnosticou 63 pessoas (4,5%) parasitadas com o *S. mansoni* (BARBOSA et al., 2015).

Além disso, sobre as ocorrências de morbidade da esquistossomose, o estado de Pernambuco apresentou outras formas clínicas graves como; hipertensão arterial pulmonar e mielorradiculopatia esquistossomótica (ARAÚJO et al., 2006; SILVA; DOMINGUES, 2011; FERREIRA et al., 2009; 2014). Através de um estudo descritivo, Almeida et al. (2013), descreveram 178 casos de hemorragia digestiva alta em um Hospital de emergência na Capital do Recife, ao longo de um período de 12 meses. Em 86% dos casos, o sangramento ocorreu devido à ruptura de varizes esofágicas, e 50% dos casos apresentavam a forma hepatoesplênica. Observou-se que a morte dentro de três meses ocorreu em 27% dos casos, devido à recorrência de hemorragia e através da impossibilidade de obtenção de atendimento no sistema público de saúde para o tratamento e erradicação das varizes esofágicas.

Outro estudo detalhado realizado em Pernambuco sobre a evolução temporal de mortes, internações hospitalares e formas clínicas graves entre os anos 1999 a 2014, no Hospital das Clínicas da Universidade Federal de Pernambuco (HC-UFPE), constatou que entre 1999 a 2013 foram quantificados 2.578 óbitos por esquistossomose e entre 2008 e 2014 foram registradas 473 internações por esta doença. Entre 1999-2014 foram identificados 1.943 casos de esquistossomose. Entre estes casos, 72,6% (n. 1411) dos indivíduos apresentavam a forma clínica hepatoesplênica, 60,8% (n. 858) apresentavam faixa etária entre 30-59 anos, sendo 58% correspondente ao sexo feminino. Entre os casos 4,6% (n. 58) tinham ascite, 43,2% (n. 556) apresentaram hemorragia digestiva alta e 39,1% (n. 489) apresentavam circulação colateral. Os padrões de fibrose avançada no fígado e muito avançada ocorreram em 65,5% (n. 793) dos casos. Entre 1999 a 2014 a curva de evolução das formas clínicas graves da esquistossomose manteve-se estável, mostrando uma tendência a diminuir a partir de 2012 (BARBOSA et al., 2016).

Por fim, estudo realizado por Martins-Melo et al. (2014), sobre as tendências de mortalidade por esquistossomose nas Regiões do Brasil durante o período de 2000 a 2011, afirmaram o que foi visto por Barbosa et al. (2016), pois demonstraram que o estado de Pernambuco apresentou a maior proporção de mortes no país, 35,2% (3085/8756 mortes), corroborando também com os dados estatísticos de Pernambuco (2014), que afirma que a região Nordeste apresenta uma média de mortalidade para esquistossomose 3 vezes maior que a média nacional (Figura 5).

Figura 5. Taxas de mortalidade por esquistossomose mansônica no Brasil, e em suas Regiões entre 2000 a 2011.



Fonte: Adaptado de Martins-Melo et al. (2014).

2.2 MORFOLOGIA DAS FASES EVOLUTIVAS DO *Schistosoma mansoni*

O trematódeo *S. mansoni* possui fases evolutivas complexas, as quais se diferenciam de acordo com o hospedeiro e o local do habitat, e são oito estágios bem distintos: ovo, miracídio, esporocistos (primário e segundário), cercárias, esquistossômulos, vermes jovens e adultos (COLLEY et al., 2014; McMANUS et al., 2018).

O ovo de *S. mansoni* mede 110 a 180 µm de comprimento (média: 150 µm) por 45 a 70 µm de largura (média: 65 µm), com um formato oval (Figura 6A), tem o pólo anterior mais delgado e o posterior mais volumoso, com um espículo saliente lateral e agudo em sua extremidade voltado para trás (REY, 2011; NEVES, 2012). Dois órgãos do verme adulto fêmea são responsáveis pela produção dos ovos: glândulas vitelínicas e ovário. O ovo, quando

liberado, ainda se encontra no estágio imaturo, caracterizado pela casca proteica contendo células vitelínicas (zigoto). Até atingir a sua maturação total, o ovo passa por 5 estádios em um período de aproximadamente 7 dias, da sua postura até chegar ao lúmen intestinal. Os ovos maduros são caracterizados por apresentarem o estágio larval, o miracídio, no seu interior. Nos tecidos, o ovo tem um tempo médio de vida de 20 dias e no meio ambiente de 2 a 5 dias (NEVES, 2012).

O miracídio representa a primeira fase larval de vida livre do parasita no meio aquático, mede 160 por 60 μm apresentando um formato oval (REY, 2002; NEVES, 2012). (Figura 6B). Sendo ainda caracterizado por apresentar placas epidérmicas ciliadas e anucleadas envolvidas na locomoção (nadam ativamente). Em sua região anterior está presente o terebratorium, o qual possui funções de sistema nervoso. Sendo responsável pela quimiotaxia, reconhecendo a presença dos caramujos no meio aquático. Nessa mesma região estão localizadas as aberturas das glândulas de penetração (centrais) e adesão (laterais) que o auxiliam na invasão tecidual do hospedeiro intermediário (moluscos). Seu sistema excretor é composto por dois pares de células denominadas em solenócitos, ou células flama, que se ligam a tubos coletores e se exteriorizam nas paredes laterais do corpo. Na sua porção posterior, os miracídios apresentam numerosas células germinativas que entrarão na sua fase ativa e darão origem a próxima fase evolutiva: os esporocistos (SILVA; NEVES; GOMES, 2008; NEVES, 2012).

Ao penetrar nos tecidos do *Biomphalaria* spp. os miracídios passam por diversas modificações estruturais. Perdem seus anexos como as glândulas de adesão e penetração, os cílios, o sistema excretor e por fim o terebratorium, resultando em uma estrutura sacular contendo as células germinativas que iniciam a sua intensa atividade mitótica (Figura 6C). Alguns dias depois, apresenta-se medindo cerca de 1,5mm de comprimento por 150μm de largura, sem formato definido e, à medida que as células se dividem, uma nova membrana superficial é formada envolvendo o tegumento, formando os esporicistos primários. Essa nova membrana contém microvilosidades e são formadas por ribossomos, mitocôndrias, retículo endoplasmático, glicogênio e lipídios, os quais estão envolvidos nas trocas de metabólitos essenciais para a nutrição dos esporocistos (SILVA; NEVES; GOMES, 2008; NEVES, 2012; BRASIL, 2014).

Os esporocistos secundários são formados a partir da formação de septos ou camadas contendo as células germinativas que continuam se dividindo assexuadamente. Esses septos são constituídos por novas membranas que envolvem os esporocistos primários e 150 a 200 camadas, sendo considerados assim, os esporocistos secundários. Em seguida, os esporocistos

secundários se deslocam para o hepatopâncreas, os tubos digestivos, e para o ovotéstis dos caramujos. Neste local, os esporocistos secundários amadurecem ocorrendo alterações morfológicas que irão formar uma nova fase evolutiva do *S. mansoni*, as cercárias (Figura 6C). Vale ressaltar, que um miracídio dará origem a cercárias de um único sexo (SILVA; NEVES; GOMES, 2008; NEVES, 2012).

A cercária representa a segunda fase larval de vida livre no meio aquático. Ao serem liberadas dos caramujos, necessitam ter alta resistência ao meio externo e alta locomoção para encontrar e penetrar no hospedeiro definitivo (Figura 6D). As cercárias são envolvidas por uma membrana sincicial de região citoplasmática anucleada. O tegumento externo é composto por duas membranas trilaminadas, sendo uma plasmática e outra basal coberta pelo glicocálix, a qual, dentre outras funções, contribui para a permeabilidade da cercária no momento da infecção (SILVA; NEVES; GOMES, 2008).

As larvas cercarianas podem chegar a atingir 500 μm de comprimento e são divididas em duas porções: a cefálica, que representa o corpo cercariano e seus anexos, e a caudal, formada por uma cauda bifurcada que auxilia na motilidade aquática. O corpo cercariano representa 200μm do comprimento total e possui na extremidade anterior uma ventosa oral e na região posterior uma ventosa ventral ou acetábulo, e as glândulas de penetração e adesão. Nessa mesma região, estão presentes células do sistema nervoso, do sistema digestivo, células germinativas que irão diferenciar os órgãos sexuais, células musculares e as papilas sensoriais que irão responder aos estímulos químicos e térmicos, refletindo no comportamento cercariano (DORSEY et al., 2002). A cauda das cercárias tem função primordial na locomoção em meio aquático e que também participa do processo de invasão da pele do hospedeiro. No parêquima tecidual são observados miócitos, neurônios e células de suporte e osmorregulatórias. As células pequenas de suporte são envolvidas por células musculares e possuem um citoplasma denso, contendo escassas mitocôndrias e retículo endoplasmático ruguso. A principal função dessas células pequenas está em impedir a morte das células musculares, mantendo a locomoção e posição de estruturas anexas das cercárias (DORSEY et al., 2002; SILVA; NEVES; GOMES, 2008).

Ao penetrar na pele dos hospedeiros definitivos, as cercárias perdem rapidamente a sua estrutura caudal. De fato, apenas o corpo cercariano tem sucesso na invasão, passando a ser denominado nesta fase de esquistossômulo (Figura 6E). O seu tubo digestivo torna-se ativo e o tegumento passa a ter um maior potencial de absorção e secreção associado ao escape do sistema imunológico do hospedeiro definitivo (SILVA; NEVES; GOMES, 2008). Da epiderme, o esquistossômulo precisa migrar para a derme e atingir a circulação sanguínea.

Para tal efeito, os esquistossômulos são atraídos por sinalizadores químicos como aminoácidos (L-arginina), peptídios e células endoteliais. Após 4 a 5 dias, os esquistossômulos chegam na pequena circulação e atingem o pulmão. Nessa etapa, os esquistossômulos antes denominados de pele, passam a ser chamados de esquistossômulos pulmonares. Os vermes coletados em cerca de 14 dias já apresentam um maior comprimento atingindo 400 μm e apresentam o ceco pigmentado, indicando a digestão das hemoglobinas (PINTO et al., 1990).

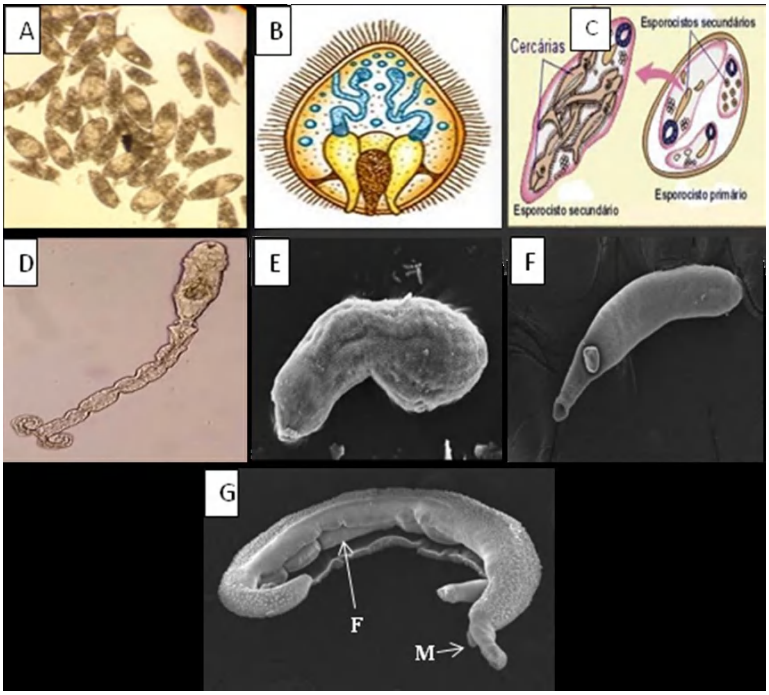
Do pulmão, os esquistossômulos ganham a grande circulação com destino ao fígado. Ao chegarem no fígado os vermes jovens de 21 dias (Figura 6F) iniciam o processo de organogênese. O verme macho apresenta dois lobos testiculares, uma curvatura dorsoventral e o canal ginecóforo em formação. A fêmea apresenta seu útero ainda primitivo e tegumento com estrias largas. Nos machos, na porção dorsal surgem protuberâncias, conhecidas como tubérculos com espinhos. Nas fêmeas, surge um ovário e inicia o desenvolvimento do oótipo. Após 35 dias, os vermes já são considerados adultos e as alterações descritas acima permanecem inalteradas (SILVA; NEVES; GOMES, 2008).

O verme macho adulto (Figura 6G) apresenta um tegumento de aspecto leitoso de comprimento médio de 1 cm e na sua região anterior estão presentes as ventosas oral e ventral (acetábulo). O sistema digestivo dos trematódeos é incompleto, iniciando-se pela boca, seguido por um ceco que se bifurcam um nível de acetábulo, percorrem as laterais do corpo e, na porção final do corpo, unem-se em um único ceco novamente. Portanto, devido à ausência de ânus, a ventosa oral tem a função de ingerir o alimento e excretar o material digerido (SILVA; NEVES; GOMES, 2008). O sistema reprodutor dos machos é constituído de 6 a 10 lóbulos testiculares localizados abaixo do acetábulo. De cada lóbulo sai um vaso eferente que se une em um canal deferente tendo o destino final no canal seminal e poro genital do canal ginecóforo; onde a fêmea é albergada e fecundada (NEVES, 2012).

As fêmeas adultas (Figura 6G) possuem um comprimento de cerca de 1,5 cm, apresentam ventosas oral e ventral mais discretos e um corpo filiforme. Tem uma coloração mais escura que os machos devido a uma maior ingestão de hemoglobina. Seu tegumento tem um aspecto liso. O sistema reprodutor é constituído por um único ovário formados por células de tamanhos distintos, os ovócitos ou oócitos. Possuem um oviduto conectado a um esfíncter denominado de ovicaptor que regula a saída dos ovócitos. Oviduto se dirige ao oótipo, formando uma passagem larga, resultando em um receptáculo seminal, sendo responsável pelo armazenamento dos espermatozoides (NEVES, 2012). Dois terços posteriores do corpo da fêmea são ocupados pelas glândulas vitelínicas, que estão envolvidas na formação do

material precursor da casca do ovo. O canal uterino geralmente apresenta um único ovo que alcança o exterior por meio do poro genital. O acasalamento entre os vermes machos e fêmeas é essencial para produção do ovo e continuidade do ciclo biológico (SILVA; NEVES; GOMES, 2008).

Figura 6. Estágios evolutivos do *Schistosoma mansoni*.



A- Ovo (**Fonte:** Araújo et al., 2020a) B- Miracídio (**Fonte:** www.coceducacao.com.br); C- Esporócitos I e II (**Fonte:** www.geocities.ws); D- Cercária (**Fonte:** Araújo et al., 2018); E- Esquitossômulos (**Fonte:** Araújo et al., 2020b); F- Vermes Jovens (Fonte: Araújo et al., 2020b) e G -Vermes Adultos, F: Fêmea e M: Macho (Fonte: Araújo et al., 2019a).

2.2.1 Ciclo biológico do *Schistosoma mansoni*

A transmissão da esquistossomose está intimamente ligada às fontes locais de água doce, que servem como habitat importante para os caramujos, hospedeiros intermediários. A implementação de esquemas de desenvolvimento industrial, agrícola e hídrico nas últimas décadas têm sido associadas ao aumento do risco de infecção nas áreas endêmicas da esquistossomose (WEBER et al., 2019). Os agentes etiológicos da esquistossomose apresentam um ciclo de vida complexo e heteroxênico, o que lhe caracteriza na Subclasse

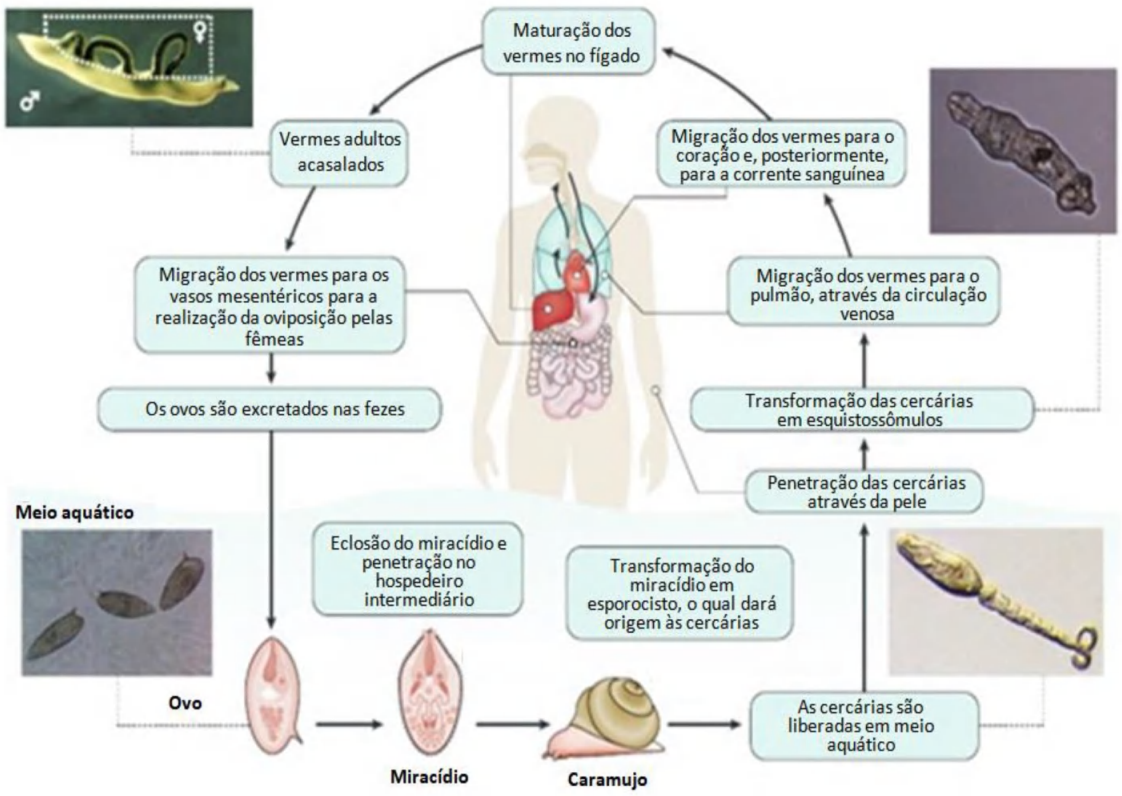
Digenea, em que obrigatoriamente o verme passa por dois ciclos de vida envolvendo um molusco aquático (da classe dos gastrópodes), os hospedeiros intermediários *Biomphalaria* spp. no Brasil, *Bullinus* spp. na África, oriente do Mediterrâneo, Ásia, Índia e Turquia, e *Oncomelania* spp. no Leste e Sudeste asiático, com o hospedeiro vertebrado definitivo, o homem (COLLEY et al., 2014; LEE et al., 2014; McMANUS et al., 2018).

Ovos do parasito são excretados junto com as fezes dos hospedeiros mamíferos infectados no ambiente externo. Em contato com a água, os miracídios eclodem, que nadam livres e ativamente (larvas ciliadas primeira fase do parasita), por até 8 h, buscando penetrar em espécies específicas de caramujos de água doce que servem como hospedeiro intermediário do parasita. Ao penetrar no molusco, o miracídio perde progressivamente seus anexos, restando apenas as células germinativas, em número de 50 a 100, que darão continuidade ao ciclo no caramujo, e se encontram na parte anterior do corpo da larva, denominando-se nessa fase de esporocistos primários ou mãe (COELHO, 1995). Os esporocistos primários entram em atividade mitótica intensa, multiplicando-se assexuadamente ou por poliembrionia para dar origem aos esporocistos II e consequentemente à formação e liberação das cercárias, e o processo pode levar de 4 a 6 semanas. A infecção ocorre quando as cercárias livres (segunda fase larval do parasita) são liberadas dos caramujos no ambiente de água doce e, por sua vez, penetram na pele de um hospedeiro mamífero (definitivo) (COLLEY et al., 2014; LEE et al., 2014; McMANUS et al., 2018). É importante ressaltar que o *S. mansoni* também pode infectar, no ambiente natural ou silvestre, outras espécies como primatas, ruminantes, roedores, marsupiais (Gambá), estes considerados hospedeiros permissivos. Epidemiologicamente são muito importantes, tornando-se responsáveis pela manutenção do ciclo do parasita no ambiente (CARVALHO; ANDRADE; CORTES, 1976; REY, 1993; GENTILE et al., 2012).

Após a penetração, as cercárias transformam-se em esquistossômulos, que migram para os pulmões via vasos sanguíneos ou linfáticos. Dentro dos pulmões, os esquistossômulos continuam a se desenvolver e são chamados de esquistossômulos pulmonares. Posteriormente migram para o sistema venoso portal, onde amadurecem sexualmente e se acasalam. Estes pares de vermes saem do sistema porta (contra o fluxo sanguíneo com auxílios das suas ventosas e espinhos presentes no dorso do verme macho), até o seu destino final, que são as veias mesentéricas superiores do intestino. Os ovos produzidos pelos vermes fêmeas (em torno de 300 unidades por dia), após quatro a seis semanas da infecção, passam por 5 estádios de maturação, e esse processo ocorre à medida que o ovo migra pela submucosa. Eles têm sucesso na passagem para o lúmen intestinal por meio da secreção de enzimas liberadas pelo

miracídio e, pelo fato da casca do ovo ser porosa, essas enzimas são facilmente transferidas para o meio externo, chegando no lúmen intestinal, onde são lançados fora junto com as fezes (COLLEY et al., 2014; LEE et al., 2014; McMANUS et al., 2018). Ainda assim, os ovos que não são liberados junto às fezes (aproximadamente 50%) ficam alojados nos órgãos do hospedeiro tais como fígado, baço e intestinos induzindo a formação de granulomas, o que contribui para a patologia da doença ou mesmo podem ocasionar formas ectópicas da esquistossomose (LIMA et al., 2017) (Figura 7).

Figura 7 - Ciclo biológico do *Schistosoma mansoni*.



Fonte: Adaptado de McManaus et al. (2018).

2.3 PATOGENIA DA ESQUISTOSSOMOSE

A patogenia da esquistossomose é resultante da resposta imune granulomatosa mediada por células do hospedeiro definitivo ao antígeno SEA do *S. mansoni*, que progride

para fibrose e, consequentemente, para hipertensão portal grave (SCHWARTZ; FALLON, 2018). Os ovos dos parasitas permanecem viáveis no fígado por cerca de 3 semanas. Primeiramente, os ovos causam uma resposta moderada do tipo Th1 aos antígenos do ovo. No entanto, geralmente essa resposta evolui de moderada para uma resposta imune do tipo Th2 com o recrutamento de eosinófilos, formação de granuloma e fibrogênese do fígado (WYNN et al., 2004; WILSON et al., 2007; SCHWARTZ; FALLON, 2018).

Embora a formação do granuloma seja de uma certa forma favorável para o hospedeiro, porque bloqueia os efeitos hepatotóxicos do SEA liberado pelos ovos do parasita retidos no órgão, esse processo pode levar à fibrose com acúmulo excessivo de proteínas do colágeno e da matriz extracelular no espaço periportal (MORAIS et al., 2008). A formação de granuloma é uma reação de hipersensibilidade tardia mediada por linfócitos T auxiliada por citocinas, como interleucina-4 (IL-4) e IL-13, enquanto IL-10, IFN-γ e um subconjunto de células T reguladoras podem limitar a patogenia induzida. O equilíbrio entre as citocinas do tipo Th1 e Th2 influencia a extensão da patologia e o desenvolvimento da fibrose (GRYSEELS; STRICKLAND, 2012; SCHWARTZ; FALLON, 2018). Os ovos são detectáveis dentro dos granulomas com a subsequente formação de fibrose periportal e perilobular acentuada, que é mais pronunciada nas espécies de parasitos *S. mansoni* e *S. japonicum* (ELBAZ; ESMAT, 2013).

As manifestações clínicas da patogenia da esquistossomose passam por fases aguda e crônica que espelham a resposta imune à infecção. Alguns pacientes também podem desenvolver complicações tardias ou sofrer sequelas de co-infecção com outros parasitas, bactérias ou mesmo com vírus (BARSOUM et al., 2013), que pode acometer vários órgãos durante suas fases, variando desde casos leves, até formas graves irreversíveis que podem culminar com a morte dos pacientes. Os achados clínicos fazem com que a sintomatologia da doença diversifique nas seguintes fases: aguda e crônica (LAMBERTUCCI, 2010; SARVEL, 2011; GRYSEELS; STRICKLAND, 2012; McMANAUS et al., 2018).

Os sintomas e a morbidade da esquistossomose mansônica dependem do número de ovos depositados pelo parasito. Desde que o ovo é colocado, até que atinja a luz intestinal, decorre um período mínimo de seis dias, tempo necessário para a maturação do ovo. Se, decorridos cerca de 20 dias, os ovos não conseguirem atingir a luz intestinal, ocorrerá a morte dos miracídios. Os ovos podem ficar presos na mucosa intestinal ou serem embolizados para o fígado (NEVES, 2012; SCHWARTZ; FALLON, 2018).

2.3.1 Forma aguda ou inicial

Essa forma pode, didaticamente, ser dividida em duas fases: a primeira fase (fase pré-postural) vai desde a penetração da cercária até um período que antecede a sua postura. Esta fase pode-se manifestar como dermatite cercariana, forma inaparente ou assintomática e forma sintomática leve e a segunda (fase postural ou pós-postural) coincide com a postura dos ovos (LAMBERTUCCI, 2010; FERRARI; MOREIRA, 2011).

2.3.2 Dermatite cercariana

O infectado pode relatar prurido e o aparecimento de uma erupção pápulo-eritrematosa no local da penetração da cercária. Esta manifestação dermatológica pode durar de 12 a 48 horas no indivíduo não sensibilizado (fenômeno de hipersensibilidade). As cercárias são detidas e destruídas na pele, o que provoca uma reação imunoinflamatória aguda, causa do prurido observado nessas situações (GRYSEELS; STRICKLAND, 2012; McMANAUS et al., 2018).

2.3.3 Forma inaparente

Acomete geralmente crianças de áreas endêmicas, que são ´´imunes``, pois a exposição a cercarias do *Schistosoma* spp. ocorrem precocemente em suas vidas. O quadro é assintomático ou geralmente passa despercebido, com uma clínica semelhante a outras doenças infecciosas, evoluindo para a fase crônica silenciosamente (McMANAUS et al., 2018).

2.3.4 Forma sintomática leve

Aparece geralmente durante a migração pulmonar e hepática dos esquistossômulos que, respectivamente, ocorrem entre o sexto e o vigésimo dias de contaminação. O infectado apresenta febre, inapetência, tosse seca, dor abdominal, náuseas, hepatomegalia dolorosa e leve esplenomegalia. Os exames parasitológicos de fezes são negativos e, nessa fase pode ser observada uma elevada eosinofilia periférica. Os sinais e sintomas têm duração de uma a três semanas, havendo nítida progressão para uma sensação de uma cura espontânea (LAMBERTUCCI, 2010; GRYSEELS; STRICKLAND, 2012).

2.3.5 Fase postural ou pós-postural

2.3.5.1 Formas crônicas

A fase crônica ou tardia inicia-se seis meses após a infecção, podendo durar vários anos, com capacidade de surgir sinais de comprometimento de diversos órgãos, com graus extremos de severidade. As manifestações clínicas variam, a depender da localização do parasito, da intensidade da carga parasitária e da capacidade de resposta do indivíduo ou do tratamento estabelecido, podendo apresentar as formas intestinal, hepatointestinal, hepatoesplênica (compensada e descompensada) (BRASIL, 2010; LAMBERTUCCI, 2010).

2.3.5.2 Forma intestinal

Coincide com a eliminação de ovos nas fezes. O paciente apresenta manifestações digestivas (surtos de diarreia intercalados por períodos de constipação). O estado geral do paciente é bom, não apresentando febre nem hepatoesplenomegalia. (GRYSEELS; STRICKLAND, 2012).

2.3.5.3 Forma hepatointestinal

O paciente apresenta o mesmo quadro clínico da forma intestinal. Ao exame físico, o paciente apresenta do fígado palpável à custa do lobo esquerdo (BRASIL, 2010).

2.3.5.4 Forma hepática

A apresentação clínica dos pacientes pode ser assintomática ou com sintomas da forma hepatointestinal. Ao exame físico, o fígado é palpável e endurecido, à semelhança do que acontece na forma hepatoesplênica. Na ultrassonografia, verifica-se a presença de fibrose hepática, moderada ou intensa (BRASIL, 2010).

2.3.5.5 Hepatoesplênica compensada

A característica fundamental desta forma é a presença de hipertensão portal, levando à esplenomegalia e ao aparecimento de varizes no esôfago. Os pacientes costumam apresentar sinais e sintomas gerais inespecíficos, como dores abdominais atípicas, alterações das funções intestinais e sensação de peso, devido o crescimento do baço que é maior que o crescimento do fígado. Ao exame físico, o fígado encontra-se aumentado, com predomínio do lobo esquerdo, enquanto o baço aumentado mostra-se endurecido e indolor à palpação (BRASIL, 2010; McMANAUS et al., 2018).

2.3.5.6 Hepatoesplênica descompensada

Inclui a forma mais grave da esquistossomose mansônica, responsável pelo óbito do paciente. Caracteriza-se por manifestações de insuficiência hepática severa, com provas funcionais denunciando reduzida capacidade fisiológica do fígado. É também nessa fase que o risco de surtos de hemorragias digestiva é alto, a isquemia hepática ajuda ainda mais a sua descompensação, podendo também estar relacionada à ação de vários outros fatores, como hepatite viral e alcoolismo. A ascite aparece na fase avançada da doença e/ou quando há associação com hepatopatias virais (LAMBERTUCCI, 2010; BRASIL, 2010; REY, 2011; McMANAUS et al., 2018).

2.4 TRATAMENTO DA ESQUISTOSSOMOSE

No que diz respeito à prevenção da evolução da doença para formas clínicas graves e morte, vale a pena notar que a droga eficaz para o tratamento específico da esquistossomose atualmente encontra-se disponível. Os estudos clínicos realizados tanto no passado quanto os mais recentes têm enfatizado que os tratamentos têm por objetivo evitar a evolução para formas hepatoesplênicas e causar a regressão de tais formas quando eles já se estabeleceram (SILVA; LEAL; DOMINGUES, 2013; BARBOSA et al., 2016).

Desde 1918, diversas drogas foram indicadas para o tratamento clínico das esquistossomoses, os primeiros tratamentos para a esquistossomose foram realizados com tartarato de potássio e antimônio, tártaro emético, seguido por dimercaptosuccinato de sódio e antimônio e di-(pirocatecol-2,4-dissulfonato) de sódio e antimônio, conhecido como estibofeno (NOVAES; SOUZA; ARAÚJO, 1999; KATZ; COELHO, 2008). Anos mais tarde, outros sais de antimônio foram introduzidos em clínicas médicas, tais como antimonilgluconato (Triostib®), antimonio-bis-pirocatransulfato de sódio (Stibofen®), antimônio tiomelato de sódio (Anthiomaline®) e gluconato de antimônio (Triostan®), sempre administrados por via parenteral (KATZ; COELHO, 2008). Os sais de antimônio, apesar de atuarem efetivamente contra as três principais espécies *S. mansoni*, *S. haematobium* e *S. japonicum*, não são mais utilizados no tratamento deste helminto, pois causam inúmeros efeitos colaterais, como deficiência ou baixo número de plaquetas e outras displasias sanguíneas (NOVAES; SOUZA; ARAÚJO, 1999). O cloridrato de 1-N-dietilamino-etil-amino-4-metil-9-tioxantona, a lucantona e o seu principal metabólito, o 1-Nb-dietilaminoetilamino- (Hidroximetil) -9-tioxantono, o hicantona, são eficazes, especificamente contra *S. mansoni* e *S. haematobium*, e também o fármaco 1- (5-nitro-2-tiazolil) imidazolidina-2-ona, oniridazol, é eficaz contra *S. haematobium* e *S. japonicum*. Esses medicamentos não são mais utilizados atualmente na terapia medicamentosa para a

esquistossomose, pois apresentam reações adversas muito críticas tais como: danos hepáticos e renais, convulsões, psicoses, alucinações visuais e auditivas, estados confusionais e outros efeitos indesejáveis no sistema nervoso central (NOVAES; SOUZA; ARAÚJO, 1999).

Richards e Foster (1969) e Baxter e Richards (1971), trabalhando na Pfizer Laboratories (Sandwich, Inglaterra), descreveram uma nova série de derivados de 2-amino-metiltetraidroquinolina, que mostraram marcada atividade esquistossomicida (KATZ et al., 2008) e o uso de oxamniquina (OXA), OXA-1,2,3,4-tetra-hidro-2 (isopropilamino) metil (-) metanol 7-nitro-6-nitro-quinolina. A OXA é ativada no *Schistosoma* por meio do mecanismo da sulfotransferase, após o que a OXA se liga ao DNA (ALBONICO et al., 2015). Esta droga foi comercialmente referida como Mansil®, que é uma combinação das palavras "mansoni" e "Brazil" onde os primeiros ensaios clínicos com esta droga foram realizados (KATZ; COELHO, 2008). A principal limitação de OXA é que não é ativa contra *S. haematobium* ou *S. japonicum*, fato que desencorajou seu uso fora da América do Sul, o único local onde o *S. mansoni* é prevalente (KATZ, 2008). Alguns casos de resistência ao fármaco OXA têm sido relatados, levando a estudos sobre sua causa, isolando cepas de *S. mansoni*, altamente refratárias. Cruzamentos genéticos entre esquistossomos sensíveis e resistentes levaram à conclusão de que a resistência a OXA ocorre por meio de traços recessivos controlados por um simples sistema gene autossômico. Assim, sugere a existência de um fator esquistossomótico essencial para converter o pró-fármaco OXA no composto ativo. Vários outros dados bioquímicos confirmam essa hipótese e preveem que uma sulfotransferase parasitária é o ativador da enzima e que a perda de sua função é a causa da resistência ao OXA (EL RIDI; TALLIMA, 2013; CIOLI et al., 2014).

Esta hipótese foi recentemente confirmada através de um mapeamento de ligação que identificou o gene da Sulfotransferase de *S. mansoni* e permitiu cristalografia, análise enzimática e análise de sua interação com o fármaco, representando uma elucidação de seu mecanismo de ação (EL RIDI; TALLIMA, 2013; CIOLI et al., 2014). Segundo Katz e Coelho (2008), a OXA mostrou um mecanismo de ação relacionado ao efeito anticolinérgico, o que aumenta a motilidade do parasita, bem como a inibição da síntese de ácidos nucleicos. Quando administrado por via oral, é mais eficaz contra vermes machos do que em vermes fêmeas (CIOLI et al., 2014).

Praziquantel (PZQ) foi descoberto em 1972, inicialmente desenvolvido para uso veterinário contra cestódeos. O PZQ é um derivado pirazina-isoquinolina, um composto quiral com um centro de carbono quiral praticamente insolúvel em água, moderadamente solúvel em etanol, mas muito solúvel em clorofórmio e dimetilsulfóxido (EL RIDI; TALLIMA, 2013;

OTHMAN; SOLIMAN, 2015; MALHADO et al., 2016). Posteriormente à sua primeira síntese, foi estudado o seu espectro de ação contra várias infecções por trematódeos e cestódeos, sendo o objetivo principal obter a dose efetiva para tratamento (40 mg/kg) de todas as espécies conhecidas de esquistossomose e algumas espécies de cestoides (KUMAR; GRYSEELS, 1994; DAYAN, 2003; OLDS, 2003; DINORA et al., 2005; CHENG et al, 2009; JAURÉGUIBERRY; CAUMES, 2010; OTHMAN; SOLIMAN, 2015).

Na época de sua introdução, como alternativa à terapia medicamentosa com OXA, o custo do PZQ era um grande obstáculo à sua distribuição em massa, mas em 1983 a empresa coreana Shin Poong entrou no mercado com um novo processo e causou uma considerável redução de preço (OLDS, 2003; CIOLI et al., 2014; OTHMAN; SOLIMAN, 2015). Atualmente, o custo médio do PZQ é de cerca de US $ 0,20 por tratamento, enquanto aproximadamente o mesmo montante é gasto para a distribuição de medicamentos (CIOLI et al., 2014). Atualmente, o PQZ está disponível comercialmente em sua forma racêmica, ou seja, misturas iguais de dois enantiômeros de uma molécula quiral (CIOLI et al., 2014; MEISTER et al., 2016). A conformação (R)-PZQ é o que tem atividade anti-helmíntica, enquanto o enantiômero (S)-PZQ é ineficaz (CAMPOS et al., 2013). Nos últimos 20 anos, o PZQ e o albendazol também têm sido usados no tratamento de doenças comuns de helmintos do sistema nervoso, neurocisticercose, em países em desenvolvimento da América Latina, Ásia e África (DOLAR et al., 2012).

As drogas anti-helmínticas atualmente comercializadas têm alta eficácia, boas margens de segurança e versatilidade de administração (REDMAN et al., 1996; DOLAR et al., 2012; CIOLI et al., 2014, OTHMAN; SOLIMAN, 2015). No entanto, apesar de seguro e eficaz contra vermes adultos de todas as espécies de *Schistosoma* spp. com importância na medicina humana, PZQ não possui efeito profilático, nas doses recomendadas não possui ação contra estágios imaturos (esquistossômulos de pele e pulmonar e vermes jovens) e ainda há relatos do surgimento de cepas de *Schistosoma* spp resistentes e/ou tolerantes ao PZQ (SABAH et al., 1986; DINIZ et al., 2014; OLIVEIRA et al., 2014; ARAÚJO et al., 2020b). Assim, limitação do PZQ pode ser evidenciada através dos registros de falha terapêutica e de baixas taxas de cura parasitária, em decorrência de pacientes tratados e que albergam simultaneamente vermes em estágios imaturos e adultos. Esse cenário é preocupante, uma vez que em áreas endêmicas é comum a infecção e/ou reinfecção, e dias após o tratamento o paciente pode apresentar morbidade recorrente, sendo prática comum o retratamento contra vermes adultos que amadureceram, colocando em risco a eficácia terapêutica do PZQ (UTZINGER et al., 2003; OLIVEIRA et al., 2014).

Após administração oral, o PZQ sofre um grande metabolismo de primeira passagem no fígado, aproximadamente 15 minutos após a administração oral (DAYAN, 2003; DINORA et al., 2005; MEISTER et al., 2016). Uma situação delicada relacionada ao tratamento com o PZQ é a sua efetividade diminuída até o 28º dia, aumentando na sexta a sétima semanas após a infecção. Assim, justifica-se uma baixa atividade no tratamento de indivíduos com a presença das diferentes fases evolutivas do parasita (JAURÉGUIBERRY; CAUMES, 2010; CIOLI et al., 2014). A ação anti-helmíntica do PZQ ainda não foi totalmente elucidada; no entanto, estudos presumem que sua atividade é devido a ação no tecido tegumentar e muscular, causando contrações no parasita que são seguidas por sua morte (DAYAN, 2003; EL RIDI; TALLIMA, 2013; CIOLI et al., 2014). Alguns autores referem-se à ação do PZQ na inibição da bomba Na^+/K^+ dos parasitas, envolvendo principalmente Ca^{2+} (NOVAES; SOUZA; ARAÚJO, 1999; EL RIDI; TALLIMA, 2013). Como resultado, aumenta a permeabilidade da membrana do helminto a cátions monovalentes e bivalentes, principalmente o cálcio (influxo de cálcio em todos os parasitas), o que leva à intensificação da atividade muscular, seguida de contração e paralisia (espasmo). Como consequência, os helmintos desprenden-se dos tecidos do hospedeiro (NOVAES; SOUZA; ARAÚJO, 1999; CIOLI et al., 2014).

As alterações estruturais induzidas por PZQ no tegumento dos vermes ocorrem na seguinte sequência: despolarização da rede microtrabecular, seguida de vacuolização e, posteriormente, erosão superficial (REDMAN et al., 1996; MENDONÇA et al., 2016; ARAÚJO et al., 2019a). Estes efeitos danificam a função muscular e a estrutura do tegumento, resultando na morte do parasita. O PZQ também inibe, em baixas concentrações, a produção de ovos pelas fêmeas do parasita (ARAÚJO et al., 2020a).

O tratamento padrão da esquistossomose no Brasil é feito com uma dose oral única de 50 mg/Kg de peso corporal que é eficaz contra *S. mansoni* (BRASIL et al., 2014). Os efeitos colaterais do PZQ são de baixa intensidade e temporários, incluindo mulheres grávidas e lactantes como dor abdominal, cefaleia e sonolência (OLDS, 2003). Para o tratamento da esquistossomose em crianças (até 15 anos), a dose habitual é 60 mg/Kg; no entanto, devido à diferença de peso (criança/adulto), a dosagem correta geralmente não é atendida, pois há poucas variações de dosagem no mercado. Outro ponto negativo da forma de dosagem do comprimido para administração a crianças, além da adaptação da dose, é o sabor amargo do medicamento. No entanto, o desenvolvimento de formas farmacêuticas contendo o PZQ como princípio ativo torna-se um desafio devido à hidrofobicidade do fármaco (BRASIL, 2014; MALHADO et al., 2016).

Quando o sistema nervoso central (SNC) sofre danos, alguns especialistas recomendam o PZQ com corticosteroides simultaneamente para o tratamento de sequelas neuroinflamatórias causadas pela esquistossomose. Recomenda-se também quando o tratamento antiparasitário induz alguma reação de hipersensibilidade, apresentando características que incluem cefaleia, hemiparesia, encefalopatia, convulsões e sinais cerebelares (JAURÉGUIBERRY; CAUMES, 2010; BERKOWITZ et al., 2015).

Atualmente o praziquantel é somente produzido no Brasil pelo Instituto de Tecnologia e Fármacos (Farmanguinhos/Fundação Oswaldo Cruz - FIOCRUZ) em comprimidos de 600 mg, nas instalações do Complexo Tecnológico de Medicamentos (CTM) e na fábrica do campus de Manguinhos (PAN AMERICAN HEALTH ORGANIZATION, 2014). A distribuição do PZQ é gratuita e repassada para as secretarias estaduais de saúde (SES), pela secretaria de vigilância em saúde (SVS), ficando disponível na rede da atenção básica a saúde dos municípios ou nas unidades de referência para tratamento da esquistossomose (BRASIL, 2010).

Por fim, a quimioterapia com PZQ é e continuará sendo a espinha dorsal dos programas de controle destinados a prevenir a morbidade e diminuir tanto a prevalência quanto a intensidade da esquistossomose (MING-GANG, 2005; GOUVRAS et al., 2013). A abordagem com melhor relação custo-benefício continuará beneficiando as populações em áreas endêmicas, à medida que o tratamento se torna mais difundido e a pesquisa operacional fornece respostas para uma melhor distribuição de medicamentos (WHO, 2016, 2019). No entanto, sem outras medidas adicionais como estratégias de controle, apenas o tratamento será incapaz de quebrar o ciclo de transmissão da esquistossomose, necessitando, assim, de vigilância contínua e quimioterapia continuada, com poucas perspectivas de atingir um ponto final (UTZINGER et al., 2013, 2015, BERGQUIST et al., 2017).

2.5 ESTRATÉGIAS DE CONTROLE DA ESQUISTOSSOMOSE

As estratégias de controle da esquistossomose consistem de métodos e aplicações corretos, exigindo um estudo prévio, buscando dados epidemiológicos e da ecologia da doença, respeitando as particularidades de cada localidade, a consolidação desses resultados permitirá o fortalecimento para a realização das estratégias de controle das doenças em etapas posteriores (FRENCH et al., 2018).

Segundo Rey (1987), segue abaixo algumas metodologias e/ou estratégias operacionais que buscam o controle da esquistossomose.

- Reconhecimento geográfico da área;
- Inquéritos malacológicos;
- Inquéritos epidemiológicos sobre a população humana (geralmente por amostragem);
- Em certas áreas, inquéritos sobre outros hospedeiros vertebrados, além do homem.
- As taxas de prevalência da esquistossomose, por grupos etários, e eventualmente a carga parasitária média desses mesmos grupos;
- As condições sanitárias locais;
- Identificação dos pontos de contato da população com as coleções de águas superficiais;
- Presença, distribuição e densidade dos moluscos hospedeiros de *Schistosoma* spp. nessas águas (identificação dos focos de transmissão local), bem como suas variações temporais;
- Os hábitos da população em relação ao uso da água e outros costumes que propiciem a transmissão da doença;
- Evolução das condições epidemiológicas e da utilização humana desses focos ao longo do ano;
- Identificação dos períodos de alta e de baixa transmissão da esquistossomose, na área, associada às variações periódicas das populações de moluscos vetores;
- Presença e significação de outros reservatórios vertebrados da endemia.

Os métodos e/ou estratégias acima mencionados variam segundo as circunstâncias, havendo, em geral, necessidade de combiná-los em um programa integrado e adaptado às condições locais. Existem também outros recursos muito favoráveis e promissores que favorecem a luta contra a esquistossomose, são eles; saneamento ambiental, educação sanitária, controle do(s) hospedeiro(s) intermediário(s) e tratamento em massa das populações que apresentam percentuais altos ou muito significativos de infecção (GOMES et al., 2012, 2014).

No requisito saneamento ambiental, pode-se esperar que a transmissão da esquistossomose seja passível de controle por meio de um saneamento adequado (GOMES et al., 2014). É primordial que os serviços de infraestrutura ou saneamento básico contenham local apropriado para suportar as fezes e urinas dos humanos, uma vez que os ovos das pessoas parasitadas são eliminados nos mesmos. Assim, impedir que os ovos lançados nos excretas entrem em contato com os corpos de água doce habitados pelos hospedeiros

intermediários. Uma redução nas infecções dos moluscos, por sua vez, pode reduzir a concentração de cercárias e, consequentemente, um menor risco de infecção humana.

Outras atividades correlacionadas ao saneamento ambiental também se tornam favoráveis no controle da esquistossomose. Rey, (1987) orienta para algumas delas, que são:

- Abastecimento de água para pequenas comunidades;
- Obras de drenagem e construção de um sistema de canalizações para águas pluviais;
- Duchas e lavanderias públicas que não distem muito das casas, mas afastem os moradores dos focos;
- Sistemas de irrigação com canalizações fechadas e mediante aspersão (evitar infestação por moluscos);
- Construção de pequenas pontes para travessia, sem risco, nos córregos e riachos contaminados;
- Drenagem de depressões naturais, brejos e pântanos e
- Construções de locais destinados à recreação (parques infantis junto às lavanderias; campos de esporte e locais para natação, situados longe dos focos de transmissão).

A educação sanitária ou mesmo educação em saúde deve focar especialmente o público escolar, para que os mesmos tornen-se multiplicadores do conhecimento, enfatizando os riscos iminentes no contato com águas com a presença de cercárias. No entanto, um pensamento cuidadoso deve considerar os fatores sociológicos do local, sendo necessário o estabelecimento de ações mitigadoras ou básicas, como água canalizada nas residências ou locais alternativos, para que as pessoas que necessitem lavar, nadar, banhar-se etc. tenham um novo recurso à disposição. Este cenário é muito importante na concepção de qualquer intervenção de controle da esquistossomose, no entanto, sabe-se que essas questões são muito mais desafiadoras do que simplesmente realizar novas instalações de lavagem, banho ou canalização de águas nas residências (ZHOU et al., 2013; MWANGA; LWAMBO, 2013; MUSUVA et al., 2014). Por exemplo, Secor (2014) relata que durante os estudos de controle da esquistossomose apoiados pela Fundação Rockefeller em Santa Lúcia, os pesquisadores descobriram que, apesar do fornecimento de água para uso doméstico, as mulheres ainda preferiam lavar suas roupas no rio, porque proporcionavam a oportunidade de interações sociais. Apesar do fracasso dessa iniciativa, a água fornecida nas residências individuais era prontamente aceita para fins domésticos e para banho, tendo também os benefícios colaterais da água limpa para a prevenção de outras doenças transmitidas por vinculação hídrica.

O controle dos hospedeiros intermediários tem como objetivo central interromper o ciclo de vida dos parasitas. Isso tem sido mais comumente feito com o uso de moluscicida químico em áreas onde ainda existem altos percentuais de prevalência, sendo então aplicada a niclosamida (WHO, 2017). No entanto, as desvantagens desse método (aplicação do moluscicida), são que o mesmo além de matar os hospedeiros intermediários dos parasitas são altamente tóxicos para outros animais da fauna aquática (peixes, anfíbios, insetos, répteis, crustáceos) (COELHO; CALDEIRA, 2016; WHO, 2017). A toxicidade para os peixes e o amarelecimento da água tratada pela niclosamida diminuem a aceitabilidade da aplicação do moluscicida nas comunidades endêmicas. Apesar desses contratempos, a aplicação de moluscicida é uma das medidas mais eficazes para o controle da esquistossomose (LI; WANG, 2017).

Segundo King et al. (2015) ao realizaram uma revisão sistemática e meta-análise para resumir as experiências de controle baseado na aplicação de moluscicida sobre as espécies *Bulinus* spp. e *Biomphalaria* spp. e o seu impacto sobre a infecção humana pelas cercárias de *Schistosoma* spp. concluíram que a aplicação do moluscicida em áreas endêmicas contribuiu significativamente para a eliminação da esquistossomose.

Em relação ao tratamento da população em massa, a administração do medicamento PZQ busca prevenir novas infecções, limitando a transmissão por meio da redução da prevalência geral na população (MING-GANG, 2005; BYGOTT et al., 2009; FAVRE et al., 2015; FACCHINI et al., 2018). No entanto, antes de ocorrer uma logística de administração maciça com o PZQ em larga escala, é necessária uma avaliação epidemiológica da comunidade, buscando saber de fato qual é a prevalência da doença e a presença dos pontos de transmissão da infecção. A prevalência é a base da estratégia recomendada para quimioterapia preventiva. Em comunidades de alto risco, com prevalência ≤ 50% detectada pelos métodos parasitológicos ou prevalência ≤ 30% pelos métodos urinários (ou mais baseada na história de hematúria), todas as crianças em idade escolar e adultos economicamente ativos são considerados de alto risco (pescadores, fazendeiros, trabalhadores de irrigação e mulheres realizando tarefas domésticas) e devem realizar tratamento preventivo. Comunidades onde a prevalência determinada pelos métodos parasitológicos é de pelo menos 10%, ou quando a esquistossomose urinária detectada tem uma prevalência inferior a 30%, é classificada como tendo risco moderado. Nessas áreas, as crianças em idade escolar, assim como aquelas dos grupos de risco especiais (mencionados acima), devem receber tratamento preventivo a cada dois anos. Apenas as crianças em idade escolar são tratadas em áreas classificadas como comunidades de baixo risco (prevalência < 10% por

métodos parasitológicos). O tratamento deve ser administrado apenas duas vezes durante o ensino fundamental. Ainda sobre a prevalência < 10% a considera um percentual limite definido como baixo risco comunitário para a propagação da esquistossomose (WHO, 2014).

O tratamento repetido durante a infância e adolescência persiste na idade adulta, reduzindo a evolução crônica da esquistossomose em áreas hiperendêmicas, aumentando a qualidade de vida. Monitoramento preciso na próxima década é essencial para controlar não apenas a morbidade, mas também a mortalidade e a gravidade da doença, fortalecendo o efeito da quimioterapia preventiva em regimes de larga escala (WHO, 2011).

Além do Brasil, com destaque para o estado de Pernambuco (OLLIARO et al., 2011; FAVRE et al., 2015; FACCHINI et al., 2018), o tratamento em massa, foi realizado pelos programas nacionais de controle da esquistossomose em vários outros países como Mali e Níger (GARBA et al., 2009), Serra Leoa (HODGES et al., 2012; SESAY et al., 2014), Kenia (ONKANGA et al., 2016) e Tanzânia (NGASALA; JUMA; MWAISWELO, 2020).

2.6 HOSPEDEIROS INTERMEDIÁRIOS DO *Schistosoma mansoni* NO BRASIL

Os hospedeiros intermediários do *S. mansoni* pertencem ao Reino Animal, Filo Mollusca, Classe Gastropoda, Subclasse Pulmonata, Ordem Basommatophora, Família Planorbidae e Gênero *Biomphalaria* (LIMA, 1995).

Atualmente, 11 espécies e uma sub espécie de *Biomphalaria* spp. foram descritas no Brasil (Figura 8). Entre estas espécies, as mais estudadas no Brasil são as hospedeiras naturais *B. glabrata*, *B. tenagophila* e *B. straminea* considerando a sua infecciosidade e transmisibilidade da doença no país (SCHOLTE et al., 2012; BRASIL, 2014). Morfologicamente, *Biomphalaria* spp. apresentam estruturas bem distintas, encobertas por uma concha de composição calcária (Figura 9).

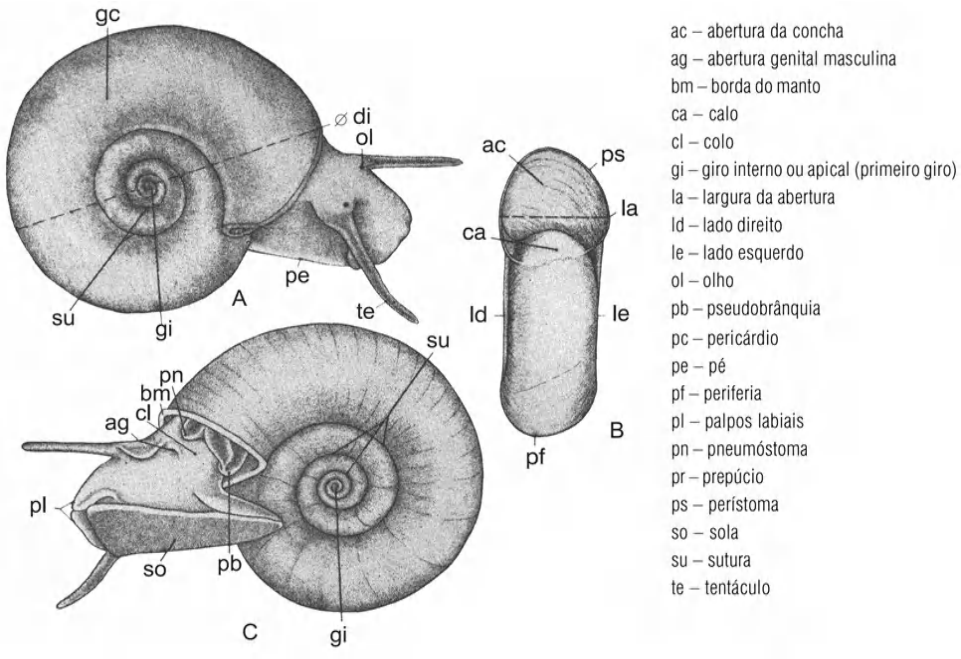
A nutrição dos *Biomphalaria* spp. é bastante diversificada são considerados onívoros (vegetais ou matéria orgânica lodo e a alimentação é realizada por uma rádula, estrutura que se situa na base da boca dos moluscos (exceto no caso dos bivalves), com a qual estes raspam o alimento. Eles são monoicos ou também denominados de hermafroditas (apresentando órgões reprodutores masculino e feminino), no entanto alcançam a fase adulta e, estando em grupos, preferem realizar fecundação cruzada, realizando oviposição (ovíparos) o ano todo (PARENSE, 1972).

Figura 8 - Espécies de *Biomphalaria* spp.

Hospedeiras naturais	*Biomphalaria glabrata* (Say, 1818)
	Biomphalaria tenagophila (Orbigny, 1835)
	Biomphalaria straminea (Dunker, 1848)
Hospedeiras potenciais	*Biomphalaria amazonica* Paraense, 1966
	Biomphalaria peregrina (Orbigny, 1835)
Não hospedeiras	*Biomphalaria intermedia* (Paraense & Deslandes, 1962)
	Biomphalaria kuhmiana (Clessin, 1883)
	Biomphalaria schrmammi (Crosse, 1864)
	Biomphalaria oligoza Paraense, 1975
	Biomphalaria occidentalis Paraense, 1981
	Biomphalaria tenagophila guaibensis Paraense, 1984

Fonte: Brasil, (2014).

Figura 9. *Biomphalaria* spp. Concha e massa cefalopodal. (A), vista pela direita, (B) vistas pela frente e (C) vista pela esquerda.



Fonte: Paraense, (2008) com modificações.

Em relação aos sistemas respiratório, digestivo e locomotor, os *Biompharia* spp. apresentam algumas particularidades, cada um deles envolve órgãos que atuam de forma favorável para a realização das funções vitais do organismo dos moluscos (BRUSKA; BRUSKA, 2007).

O sistema digestório é constituido de esôfago e estômago, subdivididos em quatros porções: 1) papo, dilatação terminal do esôfago; 2) moela, órgão fortemente musculoso usado para triturar os alimentos com o auxílio de grãos de areia; 3) piloro, término do estômago e 4) ceco, que coleta e elimina para o intestino as partículas não aproveitáveis. Entre o estômago e o ânus encontra-se o reto. O sistema locomotor de *Biompharia* spp. constitui-se pela aderência do pé ao substrato, e as contrações musculares realizadas pelo molusco permitem a propagação de uma movimentação que transcende no sentido posterior para o anterior, apresentando-se numa forma de ondas contínuas (PARAENSE 1961; 1972; BRUSKA; BRUSKA, 2007). E, por fim, quanto ao sistema respiratório, *Biomphalaria* spp. apresentam uma cavidade pulmonar (respiração em período de anidrobiose) e uma pseudobrânquia altamente vascularizados, para facilitar a respiração do molusco dentro d´água (PARAENSE 1961; 1972; BRUSKA; BRUSKA, 2007).

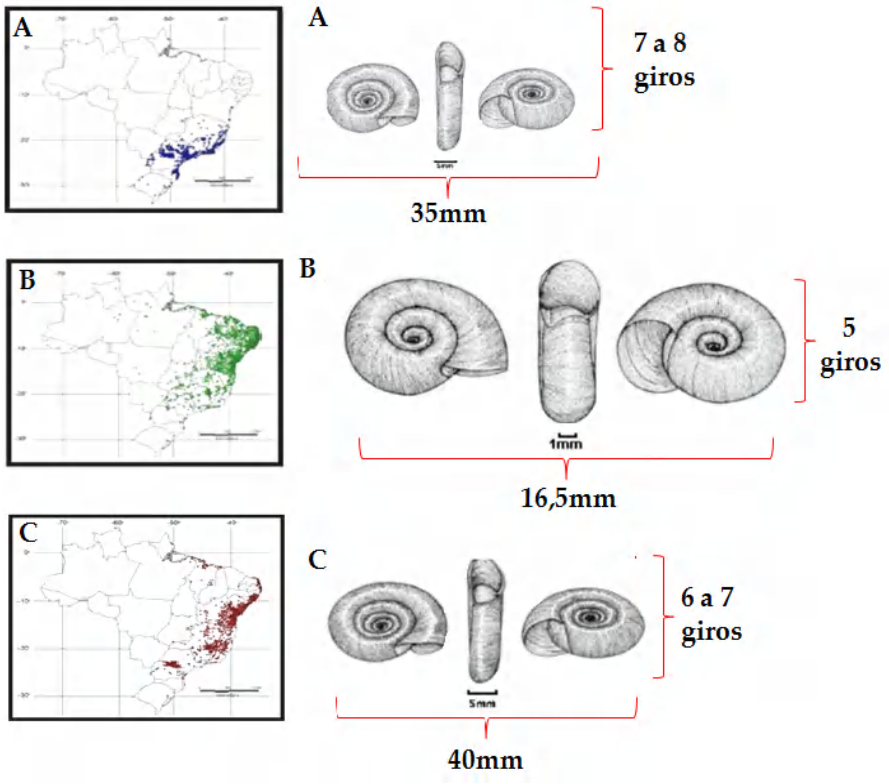
Os caramujos *B. tenagophila* se encontram ao longo da faixa litorânea do Sul do estado da Bahia até o Rio Grande do Sul. Nos estados de Minas Gerais, São Paulo, Paraná e Rio Grande do Sul, a espécie está se movendo para o sentido oeste sendo considerada muito importante na epidemiologia da esquistossomose nessas regiões. *B. tenagophila* tem importância epidemiológica no Vale do Paraíba, no estado de São Paulo (CARVALHO et al., 2018). Seu tamanho encontra-se entre 15mm a 35mm de diâmetro, com cerca de 7 a 8 giros. Apresentam uma baixa capacidade de resistência à dessecação do ambiente e por isso, a sua densidade populacional é vista ou atribuída às lâminas ou corpos d'águas permanentes (Figura 10A) (MASSARA et al., 2012; PINTO; MATI; MELO, 2013; BRASIL, 2014).

A *B. straminea* é a espécie melhor adaptada a todas as variedades de climas e condições ecológicas do território brasileiro suportando muito bem a dessecação dos ambientes aquáticos, devido ao processo de estivação, no entanto preferem águas rasas de poucas correntezas, permanentes ou temporárias (LEAL-NETO et al., 2012; BARBOSA et al., 2014a). Está espécie é a que apresentar uma maior distribuição geográfica, estando presente em quase todas as bacias hidrográficas das regiões brasileiras que vão do Norte ao Sul do país. Este cenário de ampla distribuição favorece a espécie, tornando-a epidemiologicamente a mais importante para a esquistossomose, principalmente na região Nordeste do Brasil. A sua maior representatividade encontra-se em áreas rurais, no entanto

recentemente espécimes foram encontrados em áreas litorânea, ocasionado uma maior potencialidade de infecção (BARBOSA et al., 2014a; 2014b; 2015). Em relação ao tamanho, esta espécie é a menor entre os hospedeiros naturalmente infectados com a concha medindo entre 10mm a 16mm de diâmetro e cerca de 5 giros (Figura 10B) (BRASIL, 2014).

A *B. glabrata* é o hospedeiro intermediário mais importante para a transmissão do *S. mansoni* nas Américas em função da alta suscetibilidade a diversas cepas do parasita, não morrer durante a infecção (com taxa de positividade de mais de 80% em ambiente natural), liberar no ambiente aquático em torno de 18 mil cercárias diariamente e apresentar ampla distribuição geográfica, em uma faixa contínua em todos os estados brasileiros situados entre o Rio Grande do Norte e o Paraná, com ponto isolado no Rio Grande do Sul, estando presente também em algumas áreas do Pará, Maranhão e Piauí. No Brasil, sua ocorrência está quase sempre associada à presença da esquistossomose mansônica principalmente nas áreas endêmicas (REY, 2011; BARBOSA et al., 2014b). *B. glabrata* é o maior dos moluscos pertencentes ao seu gênero, seu diâmetro de concha pode atingir de 20 a 40 mm de diâmetro e 08 a 11 mm de largura, com 6 a 7 giros arredondados (Figura 10C) (BRASIL, 2014).

Figura 10 - Distribuição geográfica e tamanhos de conchas da *Biomphalaria tenagophila* (A), *B. straminea* (B) e *B. glabrata* (C).



Fonte: Adaptado de Paraense, (1970) e Brasil, (2014).

2.6.1 Estágios embrionários dos moluscos do gênero *Biomphalaria* spp

A embriologia é o ramo da ciência que estuda o desenvolvimento embrionário dos órgãos e sistemas dos organismos vivos a partir de uma célula (HINCKE et al., 2019). Em relação a embriologia dos hospedeiros intermediários naturais (*B. glabrata*, *B. tenagophila* e *B. straminea*) do *S. mansoni*, Kawono, Nakano e Watanabe (2008) afirmam que não existem diferenciação embrionária para as respectivas espécies, sendo os estágios embrionários classificados em blástula, gástrula, trocófora, véliger e *hippo stage*.

Conforme Camey e Verdonk (1969), Kawano, Okazaki e Ré (1992) e Kawano; Nakano e Watanabe, (2008) segue abaixo as principais características embrionárias para cada estágio embrionário a partir do ovo.

O tamanho de cada ovo dos caramujos *Biomphalaria* spp. chega a medir em torno de 100 μm de diâmetro, sendo que cada desova pode conter entre algumas dezenas ou mesmo mais de 100 ovos. Os locais preferências para a oviposição das desofas são próximo à superfície ou lâmina d'água.

A partir da oviposição, inicia as múltiplas clivagens celulares até alcançar o primeiro estágio embrionário, denominado blástula, que é alcançado de 0 a 15 horas após a primeira clivagem, em seguida ocorre uma pausa por aproximadamente 10 h (Figura 11A).

O segundo estágio embrionário denominado gástrula acontece entre a 24ª a 39ª h após a primeira clivagem do ovo, caracteriza-se pelo fim da clivagem e início do crescimento, diferenciação e um pouco de movimentação celular. O tipo de gastrulação neste caramujo ocorre por invaginação. Inicialmente o embrião sofre uma mudança na sua morfologia, da forma arredondada para achatada no sentido do pólo animal para o pólo vegetativo. Na medida que as células vão se invaginando ocorre um aumento da cavidade, formando inicialmente uma abertura esférica que vai se fechando gradualmente. No final da gastrulação surgirá um dos primeiros órgãos dos moluscos, a boca (Figura 11B).

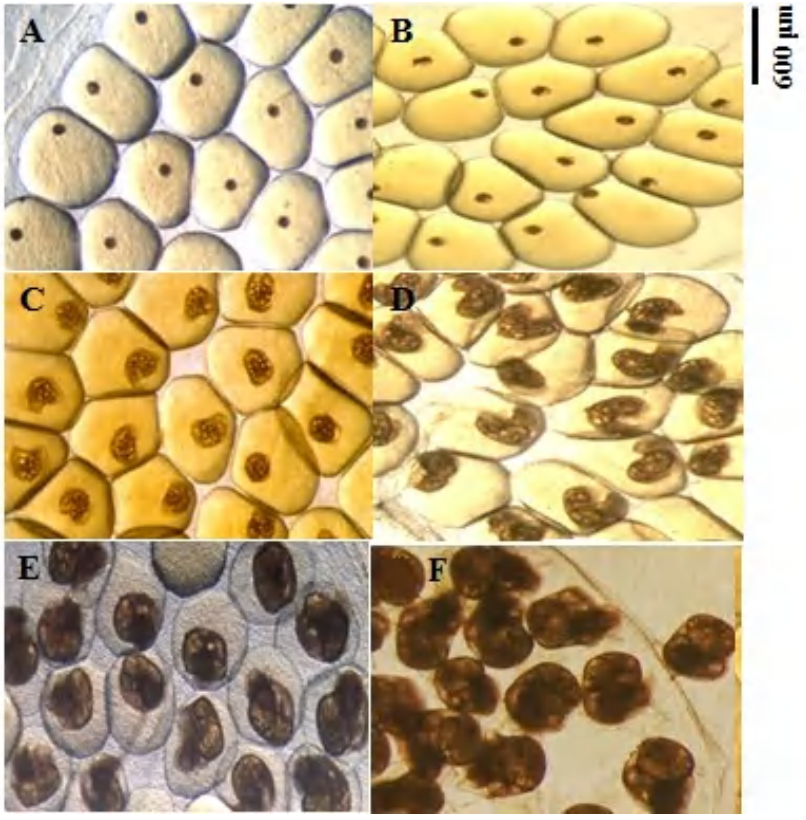
Trocófora é o terceiro estágio embrionário dos moluscos. Ocorre entre a 48ª a 87ª h após a primeira clivagem (Figura 11C). O que caracteriza o embrião neste estágio são as regiões pré-trocal e pós-trocal. Na região pré-trocal encontra-se a futura região cefálica, com a presença da placa apical, futura região dos olhos e tentáculos e da vesícula cerebral. Enquanto na região pós-trocal encontra-se a boca, situada abaixo da placa apical e na região oposta encontra-se a glândula da concha.

O estágio de véliger, ou o quarto estágio embrionários dos moluscos e caracterizado pela alta movimentação embrionária dentro do ovo e a formação quase completa de um embrião (Figura 11D), este estágio acontece entre 96ª a 111ª h após a primeira clivagem.

Por fim, temos o quinto e último estágio embrionário chamado de *hippo stage* que ocorre a partir das 120ª h da primeira clivagem. Neste estágio ocorre a formação completa do embrião. O hippo stage é caracterizado por apresentar a formação da concha que cobre parte do corpo e do pé do molusco, evidenciando o princípio da assimetria pelo desvio da concha para o lado direito, também neste último estágio onde são evidenciados os tentáculos (completamente desenvolvidos) e os olhos em suas extremidades na região das placas cefálicas (Figura 11E).

Nas condições favoráveis de temperaturas (25 ºC) a partir das 168ª após a primeira clivagem, os embriões podem eclodir das desovas, sendo então denominados caramujos jovens (Figura 11F).

Figura 11. Embriões da *Biomphalaria glabrata* em diferentes estágios evolutivos. (A) blástula, (B) gástrula, (C) trocófora, (D) véliger, (E) hippo stage e (F) caramujos jovens.



Fonte: Araújo et al. (2018a).

2.7 AGENTES MOLUSCICIDAS E ESQUISTOSSOMICIDAS PARA O CONTROLE POPULACIONAL DOS HOSPEDEIROS INTERMEDIÁRIOS E DO AGENTE ETIOLÓGICO *Schistosoma mansoni*

O controle populacional dos *Biomphalaria* spp. é uma alternativa importante para reduzir ou eliminar a esquistossomose. Atualmente, o moluscicida recomendado pela Organização Mundial da Saúde é o niclosamida apenas nos casos de alta prevalências, além disto este produto apresenta alto custo e elevada toxicidade ambiental para os representantes da fauna e flora local (OLIVEIRA-FILHO; PAUMGARTTEN, 2000; YI et al., 2005; WHO, 2017).

Por essas razões, novos agentes moluscicidas, na sua grande maioria derivados de fontes naturais, estão sendo investigados (BRACKENBURY; APPLETON, 1997; SCHALL et al., 2001; RIBEIRO et al., 2009; ALBUQUERQUE et al., 2014; ARAÚJO et al., 2018a,b,c,d; SILVA et al., 2018;2019). Muitos extratos vegetais e moléculas isoladas já foram determinados quanto ao seu potencial embriotóxico. As amidas: pelitorina, piperlonguminina e piperina não apresentaram toxicidade contra os estágios embrionários de *B. glabrata* (RAPADO et al., 2013), similarmente ocorreu com a fração aquosa e com a halitoxina da esponja marinha *Axinella viridis*, que só foram efetivas no controle dos caramujos adultos da *B. glabrata* (MIYASATO et al., 2012). Rapado et al. (2011) obtiveram resultados expressivos quando testaram extratos de diclorometano: metanol (2: 1) de folhas de *Piper cuyabanum* C.DC e *P. hostmannianum* RS nos estágios embrionários da *B. glabrata*, com 100% de inviabilidade a partir da concentração de 20 μg/mL. Oliveira-Filho et al. (2010) expuseram as massas de ovos de *B. glabrata* por 96 h ao *Euphobia milii* latex e observaram significativas malformações embrionárias a partir da concentração de 5.000 μg/mL.

Compostos de origem liquênico também já foram avaliados quanto ação moluscicida sobre *B. glabrata*. Por exemplo, Recentemente, Araújo et al. (2018b,c) relataram efeitos tóxicos e teratogênicos do ácido úsnico isolado de *Cladonia substellata* sobre os quatros primeiros estágios embrionários de *B. glabrata* (blástula, gástrula, trochóforo e veliger) em concentrações inferiores às recomendadas pela Organização Mundial de Saúde após 24 h de exposição. Além disso, as concentrações letas 50% (CL_{50}) e 90% (CL_{90}) dos estágios embrionários foram observados em 1,38 e 1,62, 3,47 e 4,45, 5,11 e 5,36 e, finalmente, 2,93 e 4,49 μg/mL, respectivamente. O extrato etéreo de *Ramalina aspera* demonstrou atividade moluscicida tanto para embriões (CL_{90} de 22,78, 24,23, 16,63 e 16,03 μg/mL para as fases de gástrula, blástula, trocófora e véliger, respectivamente), como para caramujos adultos (CL_{90}

de 8.66 μg/mL) após 24 h de exposição. As doses subletais causaram a diminuição da fertilidade nos caramujos adultos e alterações quantitativas e morfológicas em seus hemócitos. O extrato etéreo de *R. aspera* também exibiu efeito cercaricida a partir de 5.0 μg/mL (SILVA et al., 2019).

O extrato etéreo do líquen *Cladia agregata* exerceu efeitos embriotóxicos (para o estágio de blástula) a 50 e 100 μg/mL e efeitos moluscicidas (caramujos adultos) a 20 e 25 μg/mL. Enquanto o ácido barbático não exibiu embriotoxicidade para o estágio de blástula, a concentração para apresentar percentuais significativos para caramujos adultos foi igual as concentrações do extrato etéreo para embriões. Entretanto, após 60 min de exposição, a concentração de 1 μg/mL do ácido barbático apresentou atividade cercaricida (MARTINS et al., 2017).

Por fim, também foram avaliados o extrato etéreo de *Parmotrema praesorediosum* e os seus metabólitos isolados, os ácidos atranorina e praesorediósico. A embriotoxicidade (para o estágio de blástula) do extrato etéreo e do ácido praesorediósico apresentaram valores de CL_{50} de 363,07 μg/mL e 213,79 μg/mL, respectivamente. A CL_{50} estimada para os moluscos adultos tratados com o extrato etéreo foi de 102,32 μg/mL. Enquanto a atranorina não apresentou toxicidade sobre embriões e moluscos adultos (CARVALHO, 2015).

Substâncias que apresentam efeitos embriotóxicos sobre o(s) hospedeiro(s) intermediário(s) dentro de um intervalo curto de exposição são consideradas muito promissoras, uma vez que os *Biomphalaria* spp. possuem um curto ciclo embrionário (ARAÚJO et al., 2018a,b,c,d). Um único molusco atinge rapidamente a sua fase adulta e, devido as suas características fisiológicas (monóico), repovoa rapidamente os habitats aquáticos. Em apenas três meses, são capazes de produzir cerca de 10 milhões de novos descendentes (BRASIL, 2014). Especialmente no litoral do estado de Pernambuco, áreas endêmicas com a presença da espécie *B. glabrata* já foram observados, moluscos desta espécie com uma taxa de infectividade superior a 80% para *S. mansoni*, percentuais estes considerados altíssimos e de grande importância epidemiológica, uma vez que a *B. glabrata* e uma espécie que adapta-se facilmente às condições dos ambientes costeiros, a taxas de salinidade bem acima daquelas referenciadas na literatura (SILVA et al., 2006; LEAL-NETO et al., 2013).

A busca por novos compostos bioativos que possam ser utilizados como agentes esquistossomicida tem recebido mais atenção. Diversos estudos (*in vitro*, *in vivo*, pré-clínico e clínico) com novos agentes esquistossomicidas ou mesmo fármacos utilizados para outros fins

já foram ou serão avaliados e relatados para espécies de *Schistosoma* spp. (CIOLI et al., 2014; MORAES, 2015; PANIC; KEISER, 2018; LAGO et al., 2019).

No que diz respeito à atividade esquistossomicida do ácido úsnico, Salloum et al. (2012) relataram que a mortalidade de 100% dos vermes de *S. mansoni* apenas ocorreu na concentração de 200 μM após 120 h de exposição, com alterações no tegumento (aparecimento de bolhas e descamação). Outras moléculas de origem natural, como a plumbagin (5- hidroxi-2-metil-1,4-naftoquinona), a β-lapachona (3,4-di-hidro-2, 2-dimetil-2H-naftol [1,2- b] piran-5,6-diona) e o fosfato de artemisinina-naftoquina foram capazes de causar alterações profundas do tegumento, caracterizadas por descamação do tegumento, formação e ruptura de bolhas, aparência de buracos, inchaço, perda de espículas, contração dorsoventral, lesão e desintegração dos tubérculos, exposição da lâmina basal e destruição do tegumento (LORSUWANNARAT et al., 2013; AIRES et al., 2014; EL-BESHBISHI et al., 2015). Alterações na motilidade e mortalidade decorrente das alterações tegumentares também foram relatados nos estudos de Moraes et al. (2011) e Oliveira et al. (2012) ao avaliar piplatina (*Piper tuberculatum*), e *Baccharis trimera* (less) DC, óleo essencial, respectivamente. O tegumento de *S. mansoni* é um importante alvo no desenvolvimento de novos fármacos esquistossomicidas, pois faz o primeiro contato do parasita com os candidatos à fármacos, além de ser responsável por funções vitais para os parasitas, entre elas, a proteção contra ataques do sistema imunológico do hospedeiro, absorção de nutrientes, metabolismo lipídico e colesterol e na síntese de algumas proteínas (SKELLY et al., 2014; SOTILLO et al., 2015).

Sabe-se que as alterações na motilidade/viabilidade (que antecede a morte), dos vermes de *S. mansoni* expostos aos derivados naturais, semi naturais ou sintético, foram em sua maioria dose dependente do tempo (dose-resposta), estando esses relatos associados as alterações nos neurotransmissores e/ou neuromoduladores como serotonina, dopamina, acetilcolina, epinefrina, neuropeptídeos, glutamato e acetilcolinesterase (SANGSTER; SONG; DEMELER, 2005; NOËL, 2008; MARKS; MAULE, 2010).

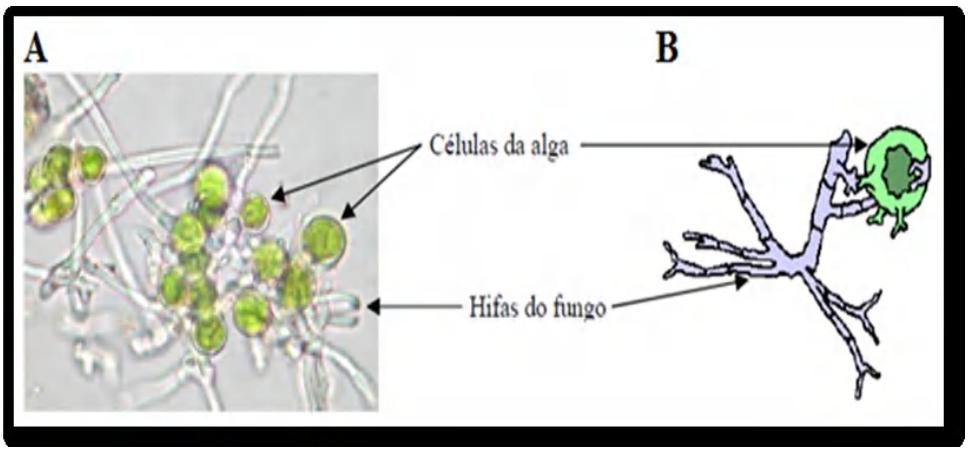
Em um estudo que envolveu a incubação de vermes de *S. mansoni* com a curcumina nas concentrações de 50 e 100 μM, Magalhães et al. (2009) observaram uma redução na motilidade/viabilidade e separação de vermes individuais machos e fêmeas após 24 h de incubação, com mortalidade de 100% e alterações tegumentares apenas na concentração de 100 μM, enquanto a exposição contínua por 120 h a 50 μM foi necessário para alcançar o mesmo porcentual de mortalidade. Estudos de Moraes et al. (2011) e Salloum et al. (2012) também demonstraram além das alterações na motilidade/viabilidade uma redução da

oviposição das fêmeas de *S. mansoni* expostas à piplatina e ao ácido úsnico, nas concentrações subletais de 9,5 e 100 μM, respectivamente. Sabe-se que os casais de vermes permanecem emparelhados no sistema sanguíneo do hospedeiro definitivo pelo resto de suas vidas (décadas), portanto, drogas que mesmo em concentrações subletais alteram a oviposição dos vermes de *S. mansoni* são extremamente importantes, considerando que os sintomas da doença nos indivíduos parasitados estão intimamente relacionados ao processo de retenção dos ovos de *S. mansoni* nos tecidos do hospedeiros definitivo, acometendo principalmente o fígado e o intestino, e nas respostas imunopatológicas do hospedeiro a eles (CIOLI et al., 2014). No aspecto epidemiológico da esquistossomose, o ovo é fundamental para o ciclo biológico do parasita, pois dentro de cada ovo existe um único miracídio, responsável por gerar até 300.000 cercárias (BRASIL, 2014).

2.8 LÍQUENS: CONCEITO GERAL, ASPECTOS MORFOLÓGICOS E USO DOS LÍQUENS NA MEDICINA POPULAR

Os líquens ou fungos liquenizados são definidos por serem simbiose, cuja associção ocorre entre um micobionte (parceiro fúngico) e um fotobionte (parceiro fotoautotrófico) (Figura 12), tornando-se complexos conjuntos de microrganismos que constituem uma fonte amplamente inexplorada de metabólitos secundários e bioativos (CALCOTT et al., 2018).

Figura 12. Filamento do micobionte (*Parmotrema trictorum*) envolvendo células do fotobionte (*Trebouxia*) visto do microscópio (A). Fungo e alga na visão simbiotica (B).



Fonte: Spielmann (2006).

Essa simbiose produz uma entidade taxonômica estável que tem um passado evolucionário. De fato, acredita-se que as simbioses semelhantes a líquens sejam uma das primeiras existentes no mundo multicelular a emergir e começar a colonizar a terra (HONEGGER, 2008). Por exemplo, hifas filamentosas associadas a cianobactérias ou algas coccoides foram encontradas preservadas no fosforito marinho da formação Doushantuo em Weng'an, sul da China entre 551 e 635 milhões de anos antepassados, sendo muito anterior à mais antiga planta terrestre conhecida, 475 milhões de anos atrás (BÜDEL; SCHEIDEGGER, 2008). Assim, a persistência dos líquens ao longo da evolução fala da eficácia da associação simbiótica.

Atualmente, os líquens existem em uma enorme diversidade de formas, sendo a estrutura morfológica do corpo (talo) dos líquens determinados pelo parceiro fúngico, cujo principal objetivo é a adaptação de maneiras diferentes para exibir o parceiro fotobionte a realizar a captura efetiva de luz para produzir os metabólitos oriundos dessa simbiose, sendo caracterizados em intracelular (primários) e extracelular (secundários) (SANDERS; DE LOS RÍOS, 2016). Os três principais tipos de crescimento são descritos em termos de sua morfologia, sendo eles; talos fruticosos, talos foliosos e talos crostosos (CALCOTT et al., 2018).

- Os talos fruticosos: semelhante a arbustos e frequentemente ramificada (Figura 13A),
- Os talos foliosos: semelhante a uma folha, com lóbulos achatados (Figura 13B),
- Os talos crostosos: formando uma crosta achatada sobre o substrato (Figura 13C).

Figura 13. Classificação dos talos liquênicos. Talos fruticoso de *Teloschistes* (A), talos folioso de *Parmotrema tinctorum* (B) e talos crostoso de *Caloplaca* (C).

Fonte: Spielmann (2006).

Na superfície da Terra estima-se que a flora liquênica domine cerca de 8% do total da vegetação, fazendo parte de diferentes culturas entre os continentes (GUO et al., 2008). No entanto, já foram descritos cerca de 1.000 metabólitos oriundos dos líquens, dos quais mais de 80% são exclusivos das classes mononucleares, aromáticas, depsidona, difenil éter e dibenzofurano (YOUSUF et al., 2014; CALCOTT et al., 2018). As regiões tropicais apresentam a maior diversidade de espécies, com até 600 espécies por hectare registradas na Costa Rica (LÜCKING et al., 2014). No entanto, as regiões temperadas também podem ser ricas em líquens, por exemplo, apesar de representar apenas 0,18% da massa terrestre do mundo, a ampla extensão geográfica, altitudinal e ecológica dos hábitats da Nova Zelândia abriga cerca de 10% das espécies de líquens da Terra (KNIGHT, 2014).

Acredita-se que certos metabólitos secundários (representados pelas substâncias liquênicas, tais como: ácidos protocetrárico, lecanórico, salazínico, atranorina, picroliquênico, fumarprotocetárico, liquesterínico, divaricático, úsnico e outros conferem tolerância ao estresse oxidativo e à exposição a poluentes (HONDA; VILEGAS, 1998; MOLNÁR; FARKAS, 2010; VAN DER WAT; FORBES, 2015). Observa-se que os policetídeos fenólicos presentes nos líquens têm fortes propriedades antioxidantes, e podem ser encontrados em níveis mais altos em líquens que experimentam maior estresse oxidativo (HIDALGO et al., 1994; WHITE et al., 2014). Esse beneficios alcançados pelos metabólitos liquênicos têm despertado interesse por possuírem diversos usos econômicos, por exemplo, o tingimento das roupas na indústria têxtil, indústria farmacêutica e cosméticos (desodorantes, cremes, xampus) e seus metabólitos secundários apresentam propriedades medicinais (MORALES; LÜCKING; ANZE, 2009)

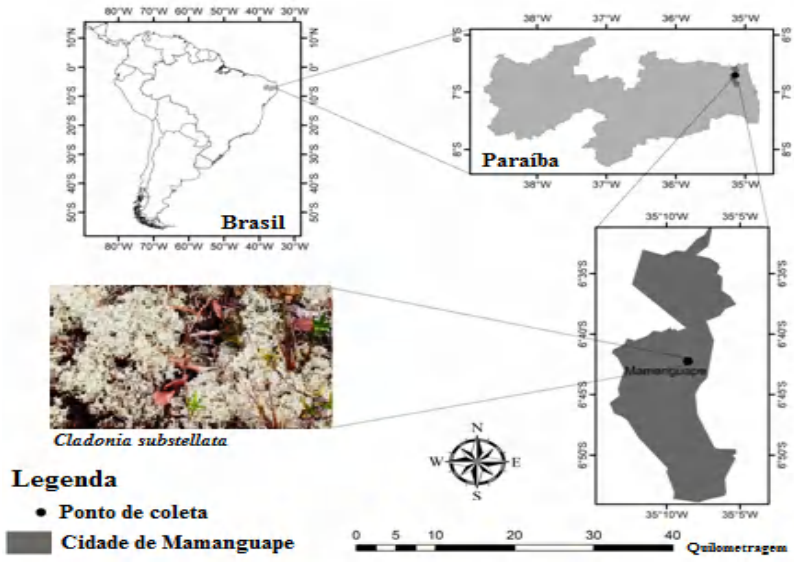
Pesquisas etnofarmacológicas enumeraram o uso de líquens e dos seus derivados na medicina popular para um amplo espectro de atividades farmacológicas, demonstrando propriedades adstringentes, laxantes, anticonvulsivantes, antieméticas, antiasmáticas, anti-inflamatórias, antibióticas, antidiarreia, doenças de pele, antiepilépticas, contra dores de garganta e de dentes e também para o tratamento de doenças cardiovasculares, respiratórias e gástricas (SHUKLA et al., 2010; YOUSUF et al., 2014). Este comportamento em captar recursos naturais está diretamente associado aos altos custos dos medicamentos sintéticos, que limitam principalmente as populações menos favorecidas economicamente sendo as mais necessitadas de atenção primária nos países pobres ou ascendestes (BALUNAS; KINGHORN, 2005).

2.8.1 *Cladonia substellata* Vanio

As Cladoniaceae são uma família de fungos formados por líquens (Ascomycotina: Lecanorales, subordem Cladoniinae), que inclui mais de 400 espécies. As cladônias são amplamente distribuídas nos países tropicais e subtropicais, reforçando que não encontradas em todos os continentes do globo Terra (AHTI, 2000; AHTI et al., 2002, 2016; PÉREZ-VARGAS et al., 2015). As espécies são facilmente reconhecidas pelas cores (por exemplo, escarlate ou branco). Algumas espécies são abundantes em certas comunidades de plantas, como aquelas nas areias brancas da Amazônia, os paramos andinos e as florestas rochosas de *Pinus occidentalis* Swartz nas montanhas da República Dominicana (AHTI, 2000; AHTI et al., 2016). Quanto a sua morfológia, apresentam talos dimórficos (uma estrutura com a combinação de dois tipos de talos crostoso-fruticoso ou escamoso-fruticoso). Normalmente os talos das Cladoniaceae crescem até 15 cm de altura, sendo que em algumas espécies podem atingir talos entre 35 a 48 cm (AHTI; STENROOS; XAVIER FILHO, 1993; AHTI, 2000; SPIELMANN, 2006).

Nos Neotrópicos, muitas espécies são difundidas, mas outras são bastante endêmicas nestes locais a exemplo da espécie *Cladonia subtellata* Vanio (1887) que encontra-se presente em diferentes regiões do Brasil, sendo elas; Sul, Centro-Oeste e Nordeste do Brasil, com destaque para o Estado da Paraíba (Nordeste), mais precisamente na cidade de Mamanguape (Figura 15), onde a espécie *C. substellata* cresce particularmente em areias expostas e nuas (AHTI; STENROOS; XAVIER FILHO, 1993; AHTI, 2000).

Figura 15 - *Cladonia substellata* na cidade de Mamanguape (Paraíba, Brasil)



Fonte: Adaptado de Araújo et al. (2019a).

Em relação aos produtos químicos produzidos decorrente do metabólismo secundário do líquen *C. subtellata* temos os ácidos: connorstictico, constístico, estístico, norstictico, criptostictic e úsnico (AHTI et al., 1993). Dentre os seis ácidos mencionados, o ácido úsnico apresenta-se como o composto majoritário para a respectiva espécie, e até mesmo para outros gêneros como *Usnea*, *Lecanora*, *Ramalina* e *Parmelia* e *Evernia*, voltando para a espécie *C. substellata* o ácido úsnico após o processo de extração, isolamento e purificação corresponde a quase 10% do peso do talo liquênico (PROKSA et al., 1994; COCCHIETTO et al., 2002).

2.8.2 Atividades biológicas, toxicidade do ácido úsnico e aplicações do sal de potássio do ácido úsnico

Dentre os metabólitos secundários de líquens, o ácido úsnico (2,6-diacetil-7,9-dihidroxi-8-9b-dimetil-1,3(2H,9α/βH) - dibenzofuranos; $C_{18} H_{16} O_7$ de cor amarela. O ácido úsnico é encontrado naturalmente em duas formas racêmicas (-) e (+), diferenciados na orientação do grupo metil angular localizado na posição 9b, sendo também atribuído ao ácido úsnico um enorme potencial farmacológico, tais como atividades gastroprotetora, imunoestimulatória, antiviral, antimicrobiana, anti-inflamatória, antiprotozoária e antitumoral (GUO et al., 2008; WHITE et al., 2014; ARAÚJO et al., 2015). No entanto, o ácido úsnico tem a desvantagem por apresentar baixa solubilidade aquosa e alta toxicidade decorrente de suas características hidrofóbicas relacionados com as suas propriedades físico-químicas decorrente da presença dos quatros grupos funcionais cetônicos e do anel furano que une os dois anéis aromáticos presente na estrutura molecular (COCCHIETTO et al., 2002; INGÓLFSDÓTTIR, 2002), recorrendo sempre aos solventes orgânicos para alcançar uma boa solubilidade (KRISTMUNDSDÓTTIR et al., 2005; JIN et al., 2013).

Em relação à toxicidade aguda oral do ácido úsnico é reportado pela empresa Sigma-Aldrich (2018) que o mesmo apresenta uma DL_{50} de 838 mg/kg para camundongos. Embora o mecanismo de toxicidade do ácido úsnico ainda não tenha sido completamente elucidado. No entanto, Han et al. (2004), Pramyothin et al. (2004) e Joseph et al. (2009) já sinalizaram que o ácido úsnico atua alterando a integridade da membrana celular (característica lipofílica da droga), permitindo a liberação de enzimas hepatoespecíficas, principalmente as transaminases. Além disso, causa a destruição da função mitocondrial (complexo I a IV da cadeia de transporte de elétrons), exibindo a perda de controle da respiração celular e da síntese de adenosina trifosfato (ATP). Esses estudos indicaram que o ácido úsnico propicia a formação de radicais livres, o que resulta em lesões nas membranas celulares e mitocondriais,

expressão de genes associados à peroxidação lipídica, ciclo de Krebs e apoptose, aumentando a produção de oxigênio reativo através da cadeia de transporte de elétrons, levando à morte celular.

Nos EUA, esses danos hepatocelulares foram confirmados após o uso do LipoKinetix®, um suplemento alimentar contendo ácido úsnico. Os usuários que consumiram este suplemento, apresentaram colapso agudo do fígado, necessitando de um deles, um transplante hepático. O outro paciente, após 8 semanas, recuperou-se devido a acompanhamento especializado (NEFF et al., 2004). Da mesma forma, dois outros usuários que consumiram três cápsulas por dia de UDP-1 (suplemento dietético com 150 mg de ácido úsnico, 525 mg de carnitina e 1050 mg de piruvato de cálcio por cápsula), desenvolveram hepatotoxicidade grave após três meses de uso. Eles exibiram insuficiência hepática fulminante. Em um dos casos, o transplante de fígado foi necessário. A análise histopatológica mostrou infiltrados linfocíticos citoplasmáticos e áreas com necrose no fígado de pacientes que usaram UDP-1 (SANCHEZ et al., 2006).

Assim, diante deste cenário vários estudos objetivaram melhorar as propriedades hidrofóbica e tóxica do ácido úsnico, tais como; preparações dos complexos ácido úsnico β-ciclodextrinas (LIRA et al., 2009), ácido úsnico-poliacrilamida (FRANCOLINI et al., 2013), nanopartículas lipídicas sólidas (SANTOS et al., 2006), encapsulado em microesferas de PLGA (RIBEIRO-COSTA et al., 2004), ácido úsnico incorporado em poliuretanos (FRANCOLINI et al., 2004; LABIB et al., 2016) e em lipossomas (SIQUEIRA-MOURA et al., 2008).

Embora as substâncias liquênicas sejam bastante mencionadas na medicina popular, conforme reportado a cima, estudos com suas moléculas bioativas quimicamente modificadas são poucos. No caso do ácido úsnico na forma de sal de potássio do ácido úsnico foi demonstrada a sua eficiência sobre caramujos adultos da *B. glabrata* (MARTINS et al., 2014), e uma maior biodisponibilidade quando comparado com sua molécula parental (ácido úsnico), quanto também uma maior inibição da invasão de metástase no câncer colorretal em modelo murino com redução macroscópica do tecido hepático e diminuição significativa do aspartato aminotransferase nas concentrações 10 e 20 mg/kg com o sal de potássio do ácido úsnico (YANG et al., 2018).

Portanto, é importante introduzir novos métodos eficazes para aumentar a taxa de solubilidade e dissolução dos candidatos a fármacos, visando melhorar sua biodisponibilidade oral, aumentando a previsibilidade da resposta e/ou reduzindo a dose (GÖKE et al., 2018;

POECHEIM et al., 2018; YANG et al., 2018; ZANOLLA et al., 2018; ARAÚJO et al., 2019b).

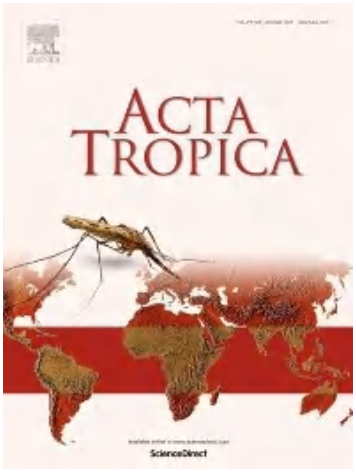
Assim, consideração o alto potencial biológico do sal de potássio do ácido úsnico, (mesmo com poucos estudos reportados na literatura) e a necessidade da busca de novas drogas esquistossomicidas e agentes moluscicidas, diantes dos dados epidemiológicos referente a doença e ampla distribuição dos hospedeiros intermediários este estudo objetiva encontrar possíveis alternativas para o controle da esquistossomose através do combate ao agente etiológico da esquistossomose, o *S. mansoni*, e ao seu hospedeiro intermediário, o molusco *B. glabrata*, utilizando como objeto de estudo o sal de potássio do ácido úsnico.

A seguir serão apresentados seis artigos científicos que representam os resultados oriundos do desenvolvimento dos objetivos proprostos.

3. RESULTADOS

3.1 ARTIGO 1

POTASSIUM USNATE TOXICITY AGAINST EMBRYONIC STAGES OF THE SNAIL *Biomphalaria glabrata* AND *Schistosoma mansoni* CERCARIAE



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Potassium usnate toxicity against embryonic stages of the snail *Biomphalaria glabrata* and *Schistosoma mansoni* cercariae

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ABSTRACT

The snail *Biomphalaria glabrata* is the most important vector for *Schistosoma mansoni*. Control of this vector to prevent the spread of schistosomiasis is currently performed with the application of a niclosamide molluscicide, which is highly toxic to the environment. Screening of substances that show embryotoxic molluscicidal potential as well as have detrimental effects on cercariae is very relevant for the control of schistosomiasis, as the efficacy of prevention of the disease is increased if it acts as a molluscicide as well as on the cercariae of *S. mansoni*. The aim of this work was to evaluate the effect of potassium usnate derived from usnic acid on different stages of embryonic development of *B. glabrata* and on *S. mansoni* cercariae. After 24 h of exposure, potassium usnate showed embryotoxic activity across all embryonic stages. The values obtained from the LC_{50} for the embryonic stages were the following: blastula 5.22 µg/mL, gastrula 3.21 µg/mL, trochophore 3.58 µg/mL, veliger 2.79, and hippo stage 2.52 µg/mL. Against *S. mansoni* cercariae, it had LC_{90} and 100% mortality at concentrations of 2.5 and 5 µg/mL in 2 h of exposure. In conclusion, this is the first report of potassium usnate toxicity on the embryonic stages of *B. glabrata* and cercariae of *S. mansoni*, and this study shows the potassium usnate as a promising agent for the control of mansoni schistosomiasis.

1. Introduction

Schistosomiasis is a parasitic disease that affects more than 260 million people in tropical and subtropical regions and causes 200,000 deaths yearly (World Health Organization, 2015). Schistosomiasis causes high morbidity and mortality, and in the severe form of the disease the patients presents periportal fibrosis, hepatosplenomegaly, reduced liver function, hemostatic and metabolic abnormalities (Lima et al., 1997; Silva et al., 2002; Leite et al., 2013, 2015; Fonseca et al., 2014).

Transmission of schistosomiasis occurs in aquatic environments contaminated with *Schistosoma* cercariae that are released by snails, the intermediate hosts of the disease (King, 2010; World Health Organization, 2015). In Brazil, due to socioeconomic problems and the presence of snails of the genus *Biomphalaria* spp., thousands of new cases and hundreds of deaths are recorded annually (Kloos et al., 2008; Caldeira et al., 2009; Barbosa et al., 2016).

The mollusk *B. glabrata* is distributed along the entire Brazilian coast, and they are also susceptible to become infected with all strains of *Schistosoma mansoni* and launch thousands of cercariae daily into the aquatic environment. In Brazil, this species is considered the main vector of the disease (Campos et al., 2002; Rey, 2014; Scholte et al., 2014). Population control of *B. glabrata* is an important alternative for reducing or eliminating schistosomiasis (Li and Wang, 2017). Currently, niclosamide molluscicide are recommended by the World Health Organization for this purpose, but these have high cost and present high environmental toxicity (Oliveira-Filho and Paumgartten, 2000; Yi et al., 2005; World Health Organization, 2017).

For this reason, alternative molluscicides derived from natural sources are being investigated (Brackenbury and Appleton, 1997; Schall

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et al., 2001; Ribeiro et al., 2009; Albuquerque et al., 2014; Silva et al., 2018). Therefore, the aim of this study was to evaluate the effect of the potassium usnate obtained from *Cladonia substellata* Vainio (1887) (Lichen) on the embryonic stages of *B. glabrata* and cercariae of *S. mansoni*.

2. Material and methods

2.1. Collection of lichen material

C. substellata Vainio samples were collected at the municipality of Mamanguape, in the state of Paraíba, Brazil (6°42′1.5″ S/35°8′3.3″W) in February 2015. A sample (voucher nº 77.474) was deposited in the UFP herbarium Geraldo Mariz. Dept. of Botany of the Federal University of Pernambuco, Recife/PE, Brazil.

2.2. Preparation of the ether extract and purification of the usnic acid

Samples (60 g) of *C. substellata* Vainio were subjected to successive extractions using diethyl ether (150 mL) in the Soxhlet apparatus at 40 °C until exhaustion (5x). Then, the extract was completely evaporated at 40 °C in a rotavaporator (Buchler Instruments, Fort Lee, NJ, USA) coupled to a 37 °C water bath (Araújo et al., 2018a).

Subsequently, isolation and purification of the single acid were performed by a chromatographic procedure: a silica gel column (70–203 mesh). The mobile phase was composed of chloroform/n-hexane (80:20 v/v) and evaporated until dryness. Identification and purity (> 98%) were evaluated through Chemical and Physicochemical analysis.

2.3. Chemical and physicochemical analysis

2.3.1. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC)

TLC: 0.1 mg samples of the organic extracts were dissolved in acetone (0.5 mL). Then 1 µL of the solution was applied to a silica gel plate (Gel 60 F254 + 366 Merck® Darmstadt, Germany) measuring 20 x 20 cm. TLC assays were run under increasing polarity conditions using the solvent system A (toluene/dioxane/acetic acid, 36: 9: 1, v/v/v) for the usnic acid. The spots were observed under ultraviolet light (256–366 nm) and visualized on the plate (Fisatom model 509) after spraying of 10% sulfuric acid and heated at 50 °C for 20 min. The composition was evaluated by the determination of the retention factor (Rf) and comparison with the standard single acid (Culberson, 1972).

HPLC: A Hitachi chromatograph (655 A-11, Tokyo, Japan) was coupled to a UV detector (655 A-11, Tokyo, Japan) at 254 nm and a reverse phase column (RP 18 MicroPack MCH-18, 300-4 mm, Berlin, Germany). The usnic acid was dissolved in methanol 0.1 g dm-3 and injected into the column 0.1 mg mL-1. The mobile phase consisted of methanol/deionized water/acetic acid (80:19.5:0.5 v/v/v) with flow of 1.0 mL min^{-1}, 0.04 attenuation at room temperature (28 ± 3 °C). The substance was identified based on its retention time (RT) and peak area when compared to standard usnic acid (Legaz and Vicente, 1983).

2.4. ^{1}H nuclear magnetic resonance (1H NMR) and infrared (IR)

The molecular structure was confirmed by proton nuclear magnetic resonance (^{1}H NMR), obtained at 300 MHz in CDCl3 (Varian UNITY spectrometer) and infrared (IR) spectroscopy were performed in a Bruker Fourier spectrometer (model IFS 66) with KBr pellets. The usnic acid standard was obtained from Sigma-Aldrich.

2.5. Synthesis of the potassium usnate

To obtain the potassium usnate, 500 mg of usnic acid was dissolved in distilled water at 40 °C, 10% potassium hydroxide was added until the solubilization of the sample at pH 11, then the sample was lyophilized (Martins et al., 2014). The structure of the potassium usnate molecule was confirmed by IR spectroscopy and proton ^{1}H NMR.

2.6. Bioassays

2.6.1. Potassium usnate toxicity assay on B. Glabrata embryos at different stages of development

Groups of 100 embryos were selected using a stereoscopic microscope (Wild M3B, Heerbrugg, Switzerland), according to the identification of Kawano et al. (1992), blastula (0–15 h Fig. 2A), gastrula (24–39 h Fig. 2B), trochophore (48–87 h Fig. 2C), veliger stages (96–111 h Fig. 2D) and hippo stage (144–168 h Fig. 2E) were placed in Petri dishes with 10 mL of potassium usnate solutions at concentrations ranging from (1; 1.5; 2; 2.5; 3; 3.5; 4; 4.5; 5; 5.5; and 6 µg/mL) blastula stage, (1; 1.5; 2; 2.5; 3; 3.5 and 4 µg/mL) gastrula stage, (1; 1.5; 2; 2.5; 3; 3.5; 4 and 4.5 µg/mL) trochophore and veliger stage (1; 1.5; 2; 2.5; 3; 3.5 and 4 µg/mL) hippo stage. A negative control group was used with only filtered and dechlorinated water (Ctrl), and a positive control group with 1.0 µg/mL niclosamide (NCL) for each embryo stage. All groups were exposed for 24 h, then embryos were washed and placed in clean plates with filtered and dechlorinated water, and observed using microscope during 8 consecutive days, in order to check their positive (hatch) or negative (death or malformation) viability (Araújo et al., 2018a). Two independent experiments were performed in triplicate, totaling 25.800 embryos.

2.6.2. Toxicity assay with S. Mansoni cercariae

S. mansoni infected *B. glabrata* adult snails (strain BH) were placed in a 400 mL beaker, submerged in 100 mL of distilled water and exposed to artificial light (60 W) for 2 h until the elimination of cercariae. The test was performed according to the description of Silva et al. (2018), with the aid of an inverted microscope (Leica DM IL Wetzlar, Germany). An estimated 100 cercariae per mL suspension was placed in a glass container and exposed to a solution of potassium usnate at concentrations of 0.5, 1, 1.5, 2.5, 5, 10, and 100 µg/mL. For the negative control, filtered and dechlorinated water was used, for the positive control a solution of niclosamide, at a concentration of 1 µg/mL. The observations of cercariae occurred at intervals of 15, 30, 60, 90 and 120 min of exposure. During exposure, the cercariae were evaluated by means of the foliowing parameters: 100% mortality of cercariae (+ + + +), mortality equal or greater than 90% (+ + +), greater than 50% (+ +), less than 50% (+) and absence of mortality (-). Two independent experiments were performed in triplicate.

2.7. Statistical analysis

Standard deviations (SD) were expressed as replicate means ± SD and the significant differences between the treatments groups were analysed using the Student's *t*-test (significance at $p < 0.05$), and were calculated using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA). The lethal concentrations (LC) required to kill 10%, 50% and 90% embryos of *B. glabrata* and *S. mansoni* cercariae were calculated by the Probit analysis with a confidence interval of 95% using the StatPlus® 2009 software (Soft Analyst, Vancouver, BC, Canada).

3. Results and discussion

3.1. Chemical analysis

The molecular structure of the usnic acid was confirmed by IR spectroscopy and ^{1}H NMR and by the similarity of the spectra reported by Martins et al. (2014). Subsequently, the synthesis of the potassium usnate was carried out and its confirmation was by IR spectroscopy and ^{1}H NMR, and the following data were obtained: IR (KBr): 3456 (*ν*

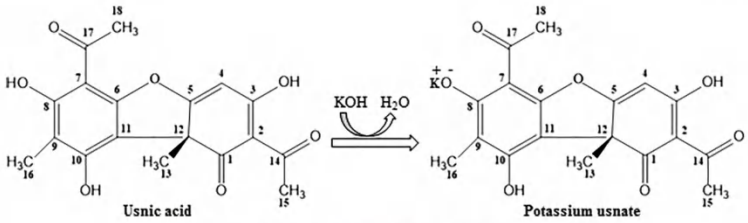

Fig. 1. Synthesis of potassium usnate.

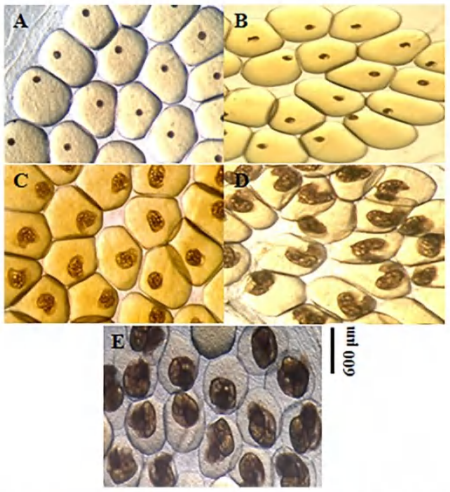

Fig. 2. *B. glabrata* different embryonic stages. (A) Blastula, (B), Gastrula, (C) Trochophore, (D) Veliger and (E) Hippo stage. All images at 40× magnification.

C−OH); 3098 (ν C−H Ar); 2988 (ν_{as} CH_3); 2929 (ν_s CH_3); 1697 (ν C=0); 1638; 1572 (ν C=C Ar.); 1446 (δ_{as} CH_3); 1380 (δ_s CH_3); 1150- 1070 (ν C−0−C) cm^{-1}. ^1H NMR (400 MH_z, acetone- d_6) δ-h:1.62 (3H, s, CH_3-13); 1.98 (3H, s, CH_3-16); 2.32 (3H, s, CH_3-15); 2.62 (3H, s, CH_3-18); 5.54 (1H, s, C-4-H); 13.44 (1H, s, C-10-OH); 14.29 (1H, s, C-3-OH) (Fig. 1).

3.2. Toxicity in embryo snails of B. glabrata exposed to potassium usnate

The results of the embryotoxic activity of potassium usnate on *B. glabrata* are presented in Table 1. In Fig. 3B–F toxic and teratogenic effects on embryo morphology are observed. Exposure to niclosamide was 100% lethal for all embryonic stages (Fig. 4A–E), but this substance presents high environmental toxicity at concentrations that lead to 100% mortality of *Artemia salina*, according to the reports by Oliveira-Filho and Paumgartten, (2000); Araújo et al. (2018b) and Silva et al. (2018), even at a concentration of 1 µg/mL. Work done in our laboratory by Martins et al. (2014), showed that potassium usnate did not present environmental toxicity to *A. salina* at concentrations below 5 µg/mL, which reinforces its potential use in population control of *B. glabrata*, as indicated in this work.

The embryonic, hippo stage (Fig. 2E) was the most susceptible to potassium usnate at all concentrations tested (Fig. 3F and Table 1). Similar results were described with the toxicity tests on embryos of *Rana nigromaculata* (frog) (Li et al., 2009), *Carassius auratus* (fish) (Wang et al., 2010) and *Physa acuta* (mollusk) (Li et al., 2014) for 1-

Table 1
Lethal concentration to all embryonic stages of *Biomphalaria glabrata* exposed to potassium usnate during 24 h and cercaricidal activity 120 min.

Embryonic stages and cercariae of *S. mansoni*	Lethal Concentration (µg/mL)		
	LC_{10}	LC_{50}	LC_{90}
Blastula	2.37 [2.28–2.45]	4.97 [4.88–5.05]	5.41 [5.32–5.49]
Gastrula	1.43 [1.39–1.47]	3.11 [3.07–3.15]	3.58 [3.54–3.61]
Trocophore	1.98 [1.93–2.03]	3.55 [3.50–3.60]	4.44 [4.39–4.49]
Veliger	1.43 [1.39–1.47]	2.67 [2.63–2.71]	3.89 [3.85–3.93]
Hippo Stage	0.85 [0.80–0.89]	2.52 [2.46–2.58]	3.81 [3.76–3.85]
Cercariae (120 min)	0.19 [0.15–0.23]	0.71 [0.67–0.75]	2.41 [2.37–2.45]

[] 95% confidence interval.

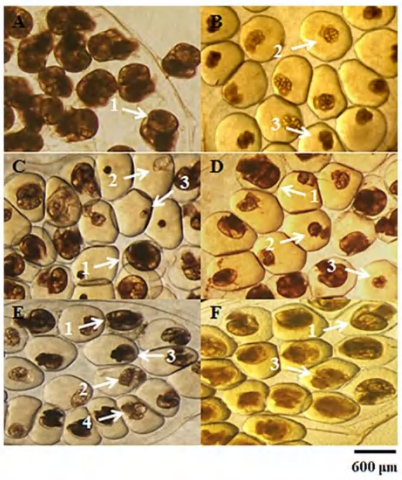

Fig. 3. *B. glabrata* embryos exposed to different concentrations of potassium usnate. (A) Normal Embryo (negative control – filtered and dechlorinated water), (B) Blastula stage (concentration 6 µg/mL), (C) Gastrula stage (concentration 2 µg/mL), (D) Trochophore stage (concentration 3 µg/mL), (E) Veliger stage (concentration 3 µg/mL) and (F) Hippo stage (concentration 1.5 µg/mL). 1: healthy embryo; 2: developmental delay; 3: dead embryo; 4: shell malformation. All images at 40× magnification.

octyl-3-methylimidazolium bromide [C_8mim]Br, where embryos in pre-hatching stages were more sensitive than in the initial stages. According to Kawano et al. (1992), this sensitivity occurs because *B. glabrata* embryos are fully formed, which could suggest that the results found in

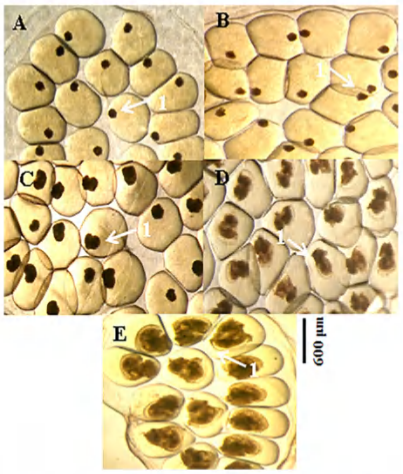

Fig. 4. *B. glabrata* embryos exposed to niclosamide (NCL) positive control at 1 µg/mL. (A) Blastula stage, (B) Gastrula stage, (C) Trochophore stage, (D) Veliger stage and (E) Hippo stage. 1: dead embryo. All images at 40× magnification.

our work are due to the greater surface area for contact, allowing for the great intramolecular action of potassium usnate.

Many plant extracts and isolated molecules have already been ascertained as to their embryotoxic potential. The amides: pellitorine, piperlonguminine and piperine showed no toxicity against the embryonic stages of *B. glabrata* (Rapado et al., 2013), similarly with the aqueous fraction and with halitoxin from the marine sponge *Axinella viridis*, which is effective only in adult control (Miyasato et al., 2012). Rapado et al. (2011), obtained expressive results when they tested extracts of dichloromethane: methanol (2:1) from leaves of *Piper cuyabanum* C.DC and *P. hostmannianum* RS on the embryonic stages of *B. glabrata*, with 100% infeasibility from the concentration of 20 µg/mL. Recently, Araújo et al. (2018a, b) reported toxic and teratogenic effects of isolated usnic acid from *Cladonia substellata* Vainio isolated on the embryonic stages of *B. glabrata* (blastula, gastrula, trochophore, and veliger) at concentrations lower than those recommended by the World Health Organization after 24 h of exposure. In addition, the LC 50 and 90% of the embryonic stages were observed in 1.38 and 1.62, 3.47 and 4.45, 5.11 and 5.36 and finally 2.93 and 4.49 µg/mL, respectively. Comparing the inviability of the embryos exposed to the usnic acid and its derivative potassium usnate, it was demonstrated that embryonic stages showed greater susceptibility to potassium usnate (Table 1), with the exception of the blastula stage. While Oliveira-Filho et al. (2010), exposed the egg masses of *B. glabrata* for 96 h to *Euphoboa milli* latex observing significant embryonic malformations from 5000 µg/mL.

Substances with embryotoxic effects on the vector within a short interval of exposure are considered promising, since *B. glabrata* has a short embryonic cycle. A single snail quickly attains its adult phase and, due to its physiological (monoecious) characteristics, rapidly repopulates aquatic habitats. In only three months, each snail is able produce about 10 million new descendants (Brasil, 2014). On the coast of the state of Pernambuco, Brazil, endemic areas of *B. glabrata* have already been observed, with a *S. mansoni* infectivity rate of greater than 80%, which is a high percentage of great epidemiological importance, since the *B. glabrata* species adapts easily to the conditions of the coastal environments, at rates well above those referenced in the literature (Silva et al., 2006; Leal-Neto et al., 2013; Barbosa et al., 2014).

Ranke et al. (2004), found that molecules with methyl and cationic groups in their composition presented a greater toxicity against luminescent bacteria (*Vibrio fischeri*); IPC-81 (leukemia cells) and C6 (glioma cells). Subsequently Li et al. (2009, 2014), and Wang et al. (2010), confirmed this hypothesis through embryotoxicity tests with a species of frog, mollusk and fish, respectively. Therefore, it is suggested that the embryotoxicity mechanism of potassium usnate is directly related to its absorption through the cellular membrane causing changes in its permeability, as described by Roberts and Costelo (2003).

Table 2
Mortality of cercariae exposed to potassium usnate in relation to exposure time.

Concentration (µg/mL)	15 min	30 min	60 min	90 min	120 min
Negative control	–	–	–	–	–
Positive control niclosamide	++++	++++	++++	++++	++++
Potassium usnate					
0.5	–	–	–	+	+
1	–	–	+	++	++
1.5	–	–	+	++	++
2.5	++	++	++	++	+++
5	++	++	+++	++++	++++
10	+++	++++	++++	++++	++++
100	++++	++++	++++	++++	++++

Negative control: filtered and dechlorinated water. Positive control niclosamide at a concentration of 1 µg/mL. Absence of lethality (−), elimination of less than 50% of cercariae (+), elimination of more than 50% of cercariae (++), lethality equal to or greater than 90% (+++) and complete elimination of cercariae (++++).

3.3. Activity of potassium usnate on S. mansoni cercariae

Screening of molluscicides that have embryotoxic potential to the mollusk vector and detrimental effects to cercariae is very relevant for the control of schistosomiasis, as the efficacy of the disease control is increased if it also acts on the cercariae of *S. mansoni* (Lima et al., 2002; El-Beshbishi et al., 2015; Martins et al., 2017; Silva et al., 2018). The evaluation of potassium usnate toxicity on *S. mansoni* cercariae is presented in Table 2. It was observed that the cercaricidal activity of potassium usnate increased proportionally with the concentration and the time of exposure, consequently leading to gradual loss in motility of the cercariae.

The cercariae were counted at the end of experiment (120 min of exposure) to obtain the final percentage of death and calculation of the LC_{10}, LC_{50} and LC_{90} (Tables 1 and 2). In Fig. 5, the graph displays the mortality of exposed cercariae to potassium usnate at the end of exposure time (120 min), in which all concentrations showed a significant mortality when compared to the negative control, with values of 15.00, 53.67, 66.67, 90.33, 100, 100, and 100% ($p < 0.0001$) at the concentrations of 0.5, 1, 1.5, 2.5, 5, 10, and 100 µg/mL, respectively.

The cercariae that were alive presented altered motility like rotation on their own axis, slow rhythm and contortions, these characteristics are completely unfavorable to the infectious capacity of the cercariae

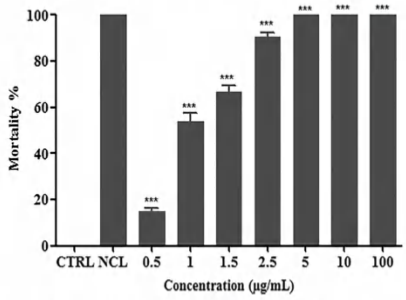

Fig. 5. Cercaricidal activity of potassium usnate against *S. mansoni* at the end of the 120 min exposure period. CTRL (filtered and dechlorinated water) and NCL (niclosamide at 1 µg/mL). The results were compared with the CTRL; *** $p < 0.0001$.

Fig. 6. *S. mansoni* cercariae exposed to potassium usnate. In A, exposed to filtered and dechlorinated water showing the preservation of the body and tail. In B, exposed for 120 min to 2.5 µg/mL potassium usnate. In C, magnified image of cercariae showing its morphological structure preserved. In D, dead cercariae exposed to 1 µg/mL of niclosamide. Magnification of the images. A and C 1.600 × . B and D 800 × .

(Brasil, 2014). Similarly, changes in the motility of *S. mansoni* cercariae were observed by Kiros et al. (2014) after 120 min of exposure to the aqueous extract of *Glinus lotoides* at concentrations 11.6 and 18.7 µg/mL, the cercariae that remained alive showed low infectivity and consequently a reduction in parasite load of 55.9% and 91.2%, respectively. We suggest that this deficiency in motility occurs due to deleterious effects on the cholinergic nervous system of cercariae, affecting the catalytic activity of acetylcholinesterase as observed by Levi-Schaffer et al. (1984), with phosphonium and phosphorane salts. The dead cercariae exposed to potassium usnate at different concentrations and commercial molluscicides niclosamide showed no morphological changes (Fig. 6B–D), unlike what was observed by Martins et al. (2017) and Silva et al. (2018) when exposing *S. mansoni* cercariae to barbatic and divaricatic acid at concentrations of 1 and 10 µg/mL, where they observed the structural separation of the cercariae in the tail and cercarial body within 30 min of exposure to both drugs, respectively. In the study of Lima et al. (2002) they tested the synthetic KOH salts of lapachol and isolapachol against *S. mansoni* cercariae and obtained 100% mortality from cercariae at a concentration of 6 µg/mL in approximately 60 min of exposure to both salts. Therefore, the application of potassium usnate as a molluscicide and cercaricide can represent an important measure to combat schistosomiasis due to its double efficacy. The comparison of these results with those of *A. salina* indicate which concentrations of potassium usnate can be used against the different embryonic stages and in the cercariae of *S. mansoni* while still being safe for the environment. The application of molluscicide is one of the most effective measures for the control of schistosomiasis (Li and Wang, 2017). According to King et al. (2015), the application of molluscicide on *Bulinus* and *Biomphalaria* spp. and their impact on human infection by *Schistosoma* spp. cercariae, they concluded that the application of molluscicide in endemic areas contributed significantly to the elimination of schistosomiasis.

4. Conclusion

The data of this work demonstrated the toxic and teratogenic effects of potassium usnate on the different embryonic stages of the mollusk *B. glabrata*. This substance was also efficient in combating *S. mansoni* cercariae. Therefore, it is suggested that potassium usnate can be used as an alternative in the population control of the vector and infective form of the pathogen at environmentally-friendly concentrations. Consequently, potassium usnate is a promising alternative in the fight against schistosomiasis.

Competing interest

The authors have no conflict of interest.

Author contributions

H.D.A. Araújo and V.L.M. Lima designed the study protocol; H.D.A. Araújo, N.H. Silva, W.N. Siqueira, M.C.B. Martins, V.L.M. Lima, A.L. Aires, M.C.P.A. Albuquerque and A.M.M.A. Melo carried out the assays and were involved in analysis and interpretation of all the data; H.D.A. Araújo, W.N. Siqueira, A.M.M.A. Melo and V.L.M. Lima contributed to drafting the manuscript and/ or critically revising the paper and intellectual content. All authors read and approved the final manuscript.

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3.2 ARTIGO 2

DATASET ON SCHISTOSOMIASIS CONTROL USING POTASSIUM ÁCIDO ÚSNICO ATE AGAINST *Biomphalaria glabrata* AT DIFFERENT DEVELOPMENTAL STAGE AND *Schistosoma mansoni* CERCARIAE

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Data Article

Dataset on schistosomiasis control using potassium usnate against *Biomphalaria glabrata* at different developmental stage and *Schistosoma mansoni* cercariae

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ABSTRACT

This text presents complementary data corresponding to schistosomiasis mansoni's vector control and toxicity on *Schistosoma mansoni* cercariae using potassium usnate. This information support our research article "Potassium Usnate Toxicity Against Embryonic Stages of the Snail *Biomphalaria glabrata* and *Schistosoma mansoni* Cercariae" [1], and focuses on the analysis of the detailed data regarding the different concentrations of potassium usnate and their efficiency to *B. glabrata* mortality and non-

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viability and *S. mansoni* cercariae mortality etiologic agent of the disease.

Specifications table

Subject area	*Chemistry, Biology*
More specific subject area	*Natural products biochemistry*
Type of data	*Tables*
How data was acquired	Stereoscopic microscope (Wild M3B, Heerbrugg, Switzerland) and an inverted microscope (Leica DM IL)
Data format	Analyzed
Experimental factors	Usnic acid purification from *Cladonia substellata* lichen and subsequently synthesis of the potassium usnate
Experimental features	Embryonic stages *B. glabrata* unviability and mortality tests and *S. mansoni* cercariae mortality assay over potassium usnate treatments were evaluated
Data source location	*Recife, Brazil*
Data accessibility	Data found in this article
Related research article	H.D.A. Araújo, A.M.M.A. Melo, W.N. Siqueira, M.C.B. Martins, A.L. Aires, M.C.P.A. Albuquerque, N.H. Silva, V.L.M. Lima, Potassium usnate toxicity against embryonic stages of the snail *Biomphalaria glabrata* and *Schistosoma mansoni* cercariae. Acta Tropica [1]

Value of the data

- The data detail the embryotoxic activity of potassium usnate on the evolutionary stages (blastula, gastrula, trophorora, veliger, and hippo stage) of *B. glabrata*, correlating the different concentrations applied to the respective stages.
- Data report a more detailed view of the malformations and death, express in LC_{10}, LC_{50}, and LC_{90}, of the different stages of development of *B. glabrata* embryos, of the original article as to their minimum concentration to reach the LC_{100} of the embryos.
- The data of different times (15, 30, 60, 90 and 120 min) allow us to infer possible time intervals to obtain effective results (LC_{10}, LC_{50} and LC_{90}) on *S. mansoni* cercariae.

1. Data

The data presented in this work provide results related to the unviability, (malformations and death) of embryos in the stages of blastula, gastrula, trophorora, veliger, and hippo stage of the *Biomphalaria glabrata* after different treatments with the potassium usnate (Table 1), as well as the potassium usnate activity on cercariae of *Schistosoma mansoni* (Table 2).

Table 1
Biomphalaria glabrata embryos unviability under different treatments with potassium usnate.

Embryonic stages	Treatment (μg/mL)	Viable embryos (%)	Unviable embryos (%)		
		Complete development	Malformed	Dead	Total unviable
Blastula	Potassium usnate				
	1.0	99.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.5	0.6 (1.0)
	1.5	99.0 ± 1.0	0.3 ± 0.5	1.0 ± 0.0	1.3 (0.5)
	2.0	98.0 ± 0.0	0.6 ± 0.5	2.0 ± 1.0	2.6 (1.5)
	2.5	75.7 ± 10.4	14.0 ± 6.0	10.3 ± 4.5	24.3 (10.5)[a]
	3.0	74.3 ± 5.8	15.3 ± 4.7	10.6 ± 1.5	25.9 (6.2)[c]
	3.5	66.6 ± 3.0	17.6 ± 4.1	15.6 ± 5.1	33.2 (9.2)[c]
	4.0	62.0 ± 6.0	13.0 ± 3.0	24.6 ± 4.0	37.6 (7.0)[c]
	4.5	55.6 ± 2.8	8.6 ± 1.5	35.6 ± 1.5	44.2 (3.0)[c]
	5.0	51.6 ± 20.1	5.0 ± 1.0	44.0 ± 18.7	49.0 (19.7)[c]
	5.5	2.3 ± 1.5	2.0 ± 1.0	95.3 ± 3.5	97.3 (4.5)[c]
	6.0	0.0	6.0 ± 4.5	93.6 ± 6.80	99.6 (11.3)[c]
	CTRL	99.3 ± 0.3	0.3 ± 0.5	0.3 ± 0.5	0.6 (1.0)
	NCL	0.0	0.0	100.0 ± 0.0	100.0 (0.0)
Gastrula	Potassium usnate				
	1.0	92.6 ± 3.7	6.0 ± 3.6	1.3 ± 1.5	7.3 (5.1)
	1.5	87.3 ± 6.1	3.6 ± 2.5	9.3 ± 3.2	12.6 (5.7)
	2.0	70.3 ± 6.4	19.0 ± 2.0[c]	10.6 ± 4.6	29.6 (6.6)[c]
	2.5	63.3 ± 4.3	15.3 ± 2.0[b]	24.6 ± 6.8[a]	39.9 (8.8)[c]
	3.0	51.5 ± 15.3	7.0 ± 8.7	41.6 ± 23.1	48.6 (31.8)[c]
	3.5	6.6 ± 2.0	3.6 ± 1.5	89.6 ± 1.5	93.2 (3.0)[c]
	4.0	0.0	0.0	100.0 ± 0.0	100.0 (0.0)[c]
	CTRL	98.6 ± 0.5	0.6 ± 0.5	0.6 ± 0.5	1.2 (1.0)
	NCL	0.0	0.0	100	100.0 (0.0)
Trocophore	Potassium usnate				
	1.0	96.0 ± .4.0	2.0 ± 1.0	1.0 ± 0.0	3.0 (1.0)
	1.5	92.6 ± 0.5	5.3 ± 1.5	2.0 ± 1.0	7.3 (2.5)
	2.0	83.0 ± 1.7	12.6 ± 4.0	2.3 ± 1.5	14.6 (15.5)[c]
	2.5	73.0 ± 5.0	23.6 ± 5.8	3.3 ± 2.3	26.9 (8.1)[c]
	3.0	67.3 ± 3.0	28.6 ± 2.5	4.0 ± 3.6	32.6 (6.1)[c]
	3.5	65.0 ± 2.0	22.3 ± 3.7	12.3 ± 3.5	34.6 (7.2)[c]
	4.0	48.0 ± 4.5	15.0 ± 7.9	37.3 ± 12.6	52.3 (20.5)[c]
	4.5	0.0	0.0	100	100.0 (0.0)[c]
	CTRL	99.0 ± 1.0	0.6 ± 0.5	0.3 ± 0.5	0.9 (1.0)
	NCL	0.0	0.0	100.0 ± 0.0	100.0 (0.0)
Veliger	Potassium usnate				
	1.0	97.6 ± 2.0	1.6 ± 0.5	1.0 ± 0.0	2.6 (0.5)
	1.5	83.0 ± 2.6	17.0 ± 1.7	0.33 ± 0.5	17.3 (2.2)[a]
	2.0	74.0 ± 6.0	18.3 ± 7.6	7.0 ± 5.0	25.3 (12.6)[c]
	2.5	74.0 ± 8.8	18.3 ± 8.9	7.6 ± 2.0	25.9 (10.9)[c]
	3.0	48.6 ± 12.1	26.3 ± 14.9	24.3 ± 2.5	50.6 (17.4)[c]
	3.5	27.6 ± 5.6	10.3 ± 5.7	62.3 ± 11.5	72.6 (17.2)[c]
	4.0	5.6 ± 2.5	5.6 ± 2.3	88.0 ± 4.5	93.6 (6.8)[c]
	4.5	0.0	4.3 ± 0.5	96.0 ± 1.0	99.6 (1.5)[c]
	CTRL	99.3 ± 0.5	0.3 ± 0.5	0.6 ± 0.5	0.9 (1.0)
	NCL	0.0	0.0	100.0 ± 0.0	100.0 (0.0)
Hippo stage	Potassium usnate				
	1.0	85.6 ± 1.5	0.6 ± 0.5	13.0 ± 3.6	13.6 (4.1)[a]
	1.5	69.6 ± 7.2	1.0 ± 1.0	28.0 ± 7.8	29.0 (8.8)[c]
	2.0	62.3 ± 2.5	0.6 ± 0.5	36.6 ± 3.0	37.2 (3.5)[c]
	2.5	53.3 ± 2.5	0.0	46.3 ± 0.5	46.3 (0.5)[c]
	3.0	46.0 ± 6.0	0.3 ± 0.5	53.3 ± 6.5	53.6 (7.0)[c]
	3.5	17.0 ± 2.6	0.0	82.6 ± 2.5	82.6 (2.5)[c]
	4.0	0.0	0.0	100.0 ± 0.0	100.0 (0.0)[c]
	CTRL	98.6 ± 0.5	0.6 ± 0.5	0.6 ± 0.5	1.2 (1.0)
	NCL	0.0	0.0	100.0 ± 0.0	100.0 (0.0)

Values expressed as mean (± standard deviation). Negative control group (CTRL) filtered and dechlorinated water only. Positive control group with 1.0 μg/mL niclosamide (NCL).

[a] The letters indicate significant differences $p < 0.5$ compared with the negative control (CTRL).
[b] The letters indicate significant differences $p < 0.001$ compared with the negative control (CTRL).
[c] The letters indicate significant differences $p < 0.0001$ compared with the negative control (CTRL).

Table 2
Lethal concentration (LC) for *S. mansoni* cercariae exposed to potassium usnate concentration 0.5, 1.0, 1.5, 2.5, 5, 10 and 100 μg/mL during 120 min.

Exposure time (min)	Lethal concentration (μg/mL)		
	LC_{10}	LC_{50}	LC_{90}
15	1.59 [1.44–1,74]	4.22 [4.07–4,37]	8.38 [8.23–8.53]
30	1.07 [0.95–1.19]	3.29 [3.17–3.41]	5.98 [5.86–6.10]
60	0.59 [0.48–0.70]	1.98 [1.87–2.09]	4.93 [4.82–5.04]
90	0.31 [0.28–0.34]	1.16 [1.13–1.19]	3.37 [3.34–3.40]
120	0.19 [0.15–0.23]	0.71]0.67–0.75]	2.41]2.37–2.45]
Niclosamide 1.0 μg/mL	nc	nc	nc

nc = not calculated. [] 95% confidence interval.

2. Experimental design, materials and methods

2.1. *B. glabrata* embryotoxicity assay

The embryotoxicity assay was performed according to the methodology described by Araújo et al. [2]. *B. glabrata* embryos were collected by depositing polyethylene sheets (10 × 10 cm^2) in aquarius. Subsequently, the embryos were packed in Petri dishes (10 mL) then, stereoscopic microscopes (Wild M3B, Heerbrugg, Switzerland) was used to evaluate and classify the embryos according to their stage of development following the methodology described by Kawano et al. [3]. The classification of the embryonic stages was determined after the first cleavage, as previously reported [4]: blastula (0–15 h), gastrula (24–39 h), trochophore (48–87 h), veliger (96–111 h) and hippo stage (144–168 h). Subsequently, groups of 100 embryos at each embryonic stage were exposed to 10 mL of potassium usnate in Petri dishes for 24 h at different concentrations as follows: blastula (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 μg/mL); gastrula (1, 1.5, 2, 2.5, 3, 3.5 and 4 μg/mL); trocophore (1, 1.5, 2, 2.5, 3, 3.5, 4 and 4.5 μg/mL); veliger (1, 1.5, 2, 2.5, 3, 3.5, 4 and 4.5 μg/mL); and hippo stage (1, 1.5, 2, 2.5, 3, 3.5 and 4 μg/mL). Only filtered and dechlorinated water (pH 7.0) was used for the negative control (CTRL) groups. The positive group was prepared to consist of 1 μg/mL niclosamide (NCL) (Bayluscide, Bayer) in filtered and dechlorinated water. After 24 h of exposure, all embryos were washed with filtered and dechlorinated water and placed in new Petri dishes containing only filtered and dechlorinated water. After 8 days the embryos were analyzed daily and classified into viable (hatching) and unviable (malformed and dead). Two independent experiments were performed in triplicate.

2.2. Toxicity assay with *S. mansoni* cercariae

Cercariae of *S. mansoni* (Strain - BH) were obtained from of *B. glabrata* adults ($n = 15$) previously infected in a laboratory with miracidia ($n = 6$). After 35 days of infection, the snails were placed in a beaker of 400 mL and submerged in 100 mL of filtered and dechlorinated water and exposed to artificial light (60 W) for 2 h until the cercariae were eliminated to obtain the cercaria suspension. The assay was performed as described in a previous work [5], the estimation of cercariae was calculated by means of an inverted microscope (Leica DM lL Wetzlar, Germany) and an aliquot of 100 cercariae/mL was transferred to a concave glass container and exposed to solutions of the potassium usnate in final concentrations of 100, 10, 5.0, 2.5, 1.5, 1.0 and 0.5 μg/mL. Cercariae from the negative and positive control groups were exposed in filtered and dechlorinated water and to niclosamide at a concentration of 1 μg/mL, respectively. The cercariae were evaluated at intervals of 15, 30, 60, 90 and 120 min after exposure. The following parameters were used for the cercaricidal evaluation: mortality of 10% (LC_{10}), 50% (LC_{50}), and 90% (LC_{90}) at different times after exposure. Two independent experiments were performed in triplicate.

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Transparency document. Supporting information

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3.3 ARTIGO 3

POTASSIUM USNATE, A WATER-SOLUBLE USNIC ACID SALT, SHOWS ENHANCED ACTIVITY AGAINST *Schistosoma mansoni IN VITRO*

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Potassium usnate, a water-soluble usnic acid salt, shows enhanced activity against *Schistosoma mansoni in vitro*

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ABSTRACT

Here, we report enhanced the *in vitro* effect of potassium usnate on coupled adult *Schistosoma mansoni* worms at different time intervals and concentrations. The evaluated schistosomicidal parameters were the following: motility, mortality, fecundity and integumentary changes, as viewed in photomicrographs. Potassium usnate was able to cause 100 and 50% mortality at 100 and 50 μM concentrations, respectively, after 24 h of exposure, while 25 and 12.5 μM concentrations caused changes in motility at 48 and 72 h, and lethality at 96 and 120 h respectively. Eggs were not detected at any of the concentrations analyzed. Photomicrographs revealed morphological tegument alterations within all periods of observation, such as swelling, blisters, dorsoventral contraction, short and curved worms. In conclusion, our results indicate that potassium usnate represents a possible candidate for a new drug in the control of schistosomiasis.

1. Introduction

Schistosomiasis is an infection caused by blood helminths of the genus *Schistosoma*, which is endemic in 78 countries and territories and affects more than 220 million people. It causes about 200,000 deaths a year, by disrupting the urogenital, hepatosplenic and gastrointestinal tract systems (World Health Organization, 2019). *S. mansoni* is the most prevalent species in the world, and is the only one found in Central and South America, including Brazil (Katz, 2008; Noya et al., 2015; Chuah et al., 2019). Transmission of schistosomiasis occurs in aquatic environments contaminated with cercariae released from intermediate hosts (McManus et al., 2018). Praziquantel (PZQ) is the only drug for schistosomiasis treatment in the world but it does not present an totally effective parasitic cure (100%) on *S. mansoni* worms in endemic areas, and there have even been sporadic reports of resistance to PZQ (Cioli et al., 2014; Oliveira et al., 2014). For this reason, natural, semi-synthetic or synthetic products are promising sources in the search for new schistosomicidal agents (Salloum et al., 2012; Aires et al., 2014; Silva et al., 2018).

Usnic acid is a secondary metabolite found in several lichen species of the genus *Cladonia*, *Usnea*, *Lecanora*, *Ramalina*, *Parmelia* and *Evernia* with promising antiparasitic activities reported (Ingólfsdóttir, 2002; Carvalho et al., 2005; Salloum et al., 2012; Luz et al., 2015) and recently molluscicide (Araújo et al., 2018a,b). However, usnic acid has the disadvantage of presenting low aqueous solubility and high toxicity due to its hydrophobic characteristics related to its physical and chemical properties (Ingólfsdóttir, 2002; Jin et al., 2013). Therefore, it is important to introduce new, effective methods to increase the solubility and dissolution rate of drug candidates in order to improve their oral bioavailability, to increase the predictability of responses, and/or to reduce dosage (Yang et al., 2018; Araújo et al., 2019). Therefore, the objective of this study was to evaluate the effect of potassium usnate, a water-soluble usnic acid salt on coupled adult *S. mansoni* worms, evaluating the parameters of motility, mortality, fecundity and tegument alterations.

2. Materials and methods

The purification of usnic acid and synthesis of potassium usnate followed the protocol detailed by Araújo et al. (Araújo et al., 2018a,c; 2019). Briefly, an ethereal extract of *C. substellata* was fractionated in a silica gel column (70–230 mesh), eluted in a chloroform hexane solvent system (80:20 v/v), evaporated to dryness and monitored by Thin-Layer Chromatography (TLC) and High-Performance Liquid Chromatography

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(HPLC - purity ≥ 98%). Subsequently, 5% potassium hydroxide was added until complete solubilization of the usnic acid. Confirmation of the molecular structure for both single and potassium usnate was performed by Infrared (IR), Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy and elemental analysis.

The experiments were conducted with the approval of the Ethics Committee on Use and Animal Experimentation (CEUA) of the Biosciences Center of the Federal University of Pernambuco (CB -UFPE) (nº 23076.015163/2017-65). *Swiss Webster* mice, weighing 28 ± 2 g, were obtained and maintained at the Laboratory of Immunopathology Keizo Asami (LIKA) of UFPE. The animals were kept at a temperature of 20 ± 2 °C and a light/dark cycle of 12 h with free access to feed (libitum/Purina®, São Paulo - SP) and dechlorinated and filtered water. Subsequently, the mice were infected percutaneously with 120 *S. mansoni* cercariae (strain: BH - Belo Horizonte, Brazil) (Olivier and Stirewalt, 1952). Mice were euthanized by cervical dislocation 60 days after infection, following the methodology described by Smithers and Terry (1965). Potassium usnate was dissolved in RPMI 1640 medium at pH 7.4 containing 20 mM HEPES and supplemented with penicillin (100 µg/mL), streptomycin (100 µg/mL) and 10% fetal bovine serum. Each well of a tissue culture dish received 2 mL of RPMI medium and two coupled adult worms were added to each well in quadruplicate (16 pairs of worms per concentration). The plates were incubated for 2 h at 37 °C in a humid atmosphere containing 5% CO_2. After this time, potassium usnate (solubilized in RPMI 1640 culture medium) was added to the culture medium to reach final concentrations of 100, 50, 25 and 12.5 µM. The worms were kept for 120 h and mortality was assessed daily. Negative and positive control groups were formed by only coupled worms incubated in supplemented RPMI 1640 medium and coupled worms exposed to 10 µM PZQ in supplemented RPMI 1640 medium, respectively. Two independent experiments. were performed in quadruplicate (Lorsuwannarat et al., 2013).

The motility and mortality of *S. mansoni* adult worms were determined according to the viability scores proposed by Silva et al. (2018), i.e. score 0, complete absence of motion and integument with or without color changes; score 1, movement present only in the extremities or in only one of the extremities (anterior and/or posterior region), with absence of peristalsis of the internal organs and no adherence of the suckers; score 2, reduced movement present throughout the body, peristalsis of internal and suctioning organs; and score 3, typical movement present, exhibiting peristalsis of internal organs, suction cups in movement, adhering to the bottom or sides of the culture plate; the latter being descriptions of worms of the negative control. The treatment was considered lethal when it was not possible to observe movement of the parasites for up to 2 min, with specimens being monitored and evaluated through an inverted microscope (Leica Microsystems, DM IL Wetzlar, Germany) at intervals of 24, 48, 72, 96 and 120 h after exposure to potassium usnate and Praziquantel (positive control group).

2.1. Statistical analysis

Numerical data were analyzed with Graphpad Prism 5 software (GraphPad Software, Inc., La Jolla - CA, USA) and are expressed as mean ± standard deviation (SD). Statistical differences were determined by using one-way analysis of variance (ANOVA) in conjunction with Turkey's test for single-step multiple comparisons. The concentration required to kill 50% (EC_{50}) in 24 h was calculated by program CompuSyn versão 1.0 (Chou and Martin, 2005).

3. Results and discussion

Purified usnic acid was obtained from an ethereal extract from *C. substellata*. After the modification of the usnic acid to form potassium usnate, the sample was again analyzed by ^{1}H NMR and IR (see supplementary data) and the spectroscopic data obtained were similarly

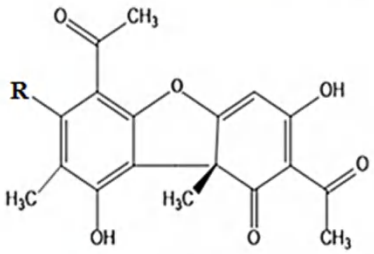

Fig. 1. Chemical structure of usnic acid. R = HO (usnic acid), R = KO (potassium usnate).

reported by Araújo et al. (2018c, 2019) (Fig. 1). Although usnic acid be a lichen metabolite, it's synthesis is easily performed by biotechnological methods, as chemo-enzymatic assays with commercially available reagents (Pereira et al., 1995; Hawranik et al., 2009; Martins et al., 2017) and potassium usnate can be produced in large scale for use commercial by a simple acid-base reaction.

The search for new bioactive compounds that can be used as antiparasitics has received more attention. Several studies (*in vitro*, *in vivo*, preclinical and clinical) with new schistosomicidal agents or even drugs used for other purposes have already been or will be evaluated and reported for species of *Schistosoma* spp. (Cioli et al., 2014; Moraes, 2015; Panic and Keiser, 2018; Lago et al., 2019). Fig. 2 expresses the viability kinetics of *S. mansoni* adult worms exposed to potassium usnate at different concentrations and time intervals. The worms of the negative control group showed viability of healthy worms, presenting typical movement including peristaltic movement of the internal organs along the body and suction cups in movement or adhered to the bottom or lateral sides of the plate, coupling at all intervals and without integumentary changes (score 3) (Fig. 3A). At 24 h, potassium usnate at 100 and 50 µM caused 100 and 50% complete absence of movements (score 0), respectively. The remaining 50% of the latter concentration featured worms with movement only at the extremities or only at one extremity with absence of peristalsis of the internal organs and no movement of the suction cups (score 1). Lethality was observed at 50 µM after 48 h of incubation. Integumentary and behavioral changes were also observed for both concentrations at the respective lethality intervals, with coupled and separate worms being observed (Fig. 3B and C, respectively). Meanwhile, the 25 µM concentration presented different viability scores: worms with reduced motility throughout the body and peristalsis of the internal organs (score 2) and score 1 at the first observation interval, respectively, yet resulted in score 0 for all worms after 96 h, with integumentary alterations and separation of the worms (Fig. 3D). Meanwhile, the concentration of 12.5 µM was able to

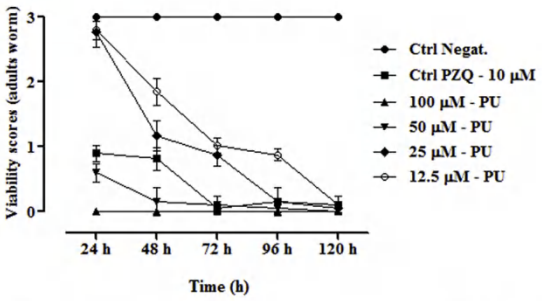

Fig. 2. Effect of different concentrations of potassium usnate (PU) on the viability of *Schistosoma mansoni* adult worms. Mean values of viability were calculated using a viability scores.

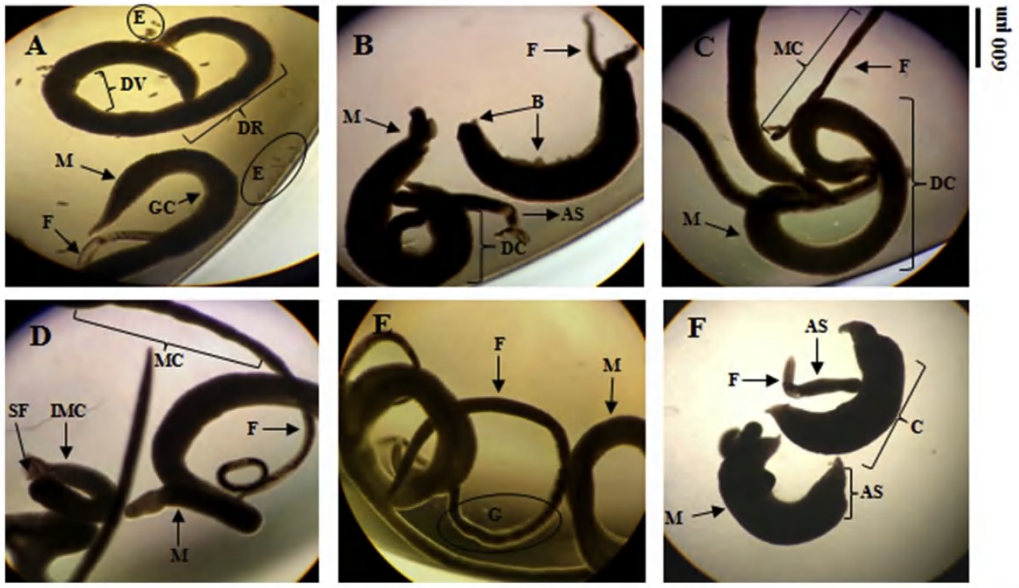

Fig. 3. A-F. Coupled adult *S. mansoni* worms. A. Negative control group, parasites incubated and maintained in supplemented RPMI 1640 medium for 120 h. Intact surface and morphological topography without structural changes in male (M), female (F) worms. It is possible to observe the gynecophoric canal (GC) and dorsal (DR) and ventral (DV) regions. In relation to oviposition, eggs (E) were observed only in the negative control group. B. Worms exposed to 100 μM potassium usnate for 24 h, showing dorsoventrally curved (DC) male worms with the presence of bubbles (B) and swollen integument (AS) in the females. C. Worms exposed to 50 μM potassium usnate for 48 h, showing muscular contractions (MC) in the anterior region of the female and median dorsoventral curvature (DC) in male worms. D. Coupled worms exposed to 25 μM potassium usnate for 96 h, male worm exhibiting intense muscle contraction (IMC) and in a spiral format (SF) and muscular contractions (MC) in the posterior portion of the female. E. Coupled worms exposed to 12.5 μM potassium usnate for 120 h, granular anterior region (G) in female worm and separation of coupled. F. Worms exposed to 10 μM Praziquantel (PZQ - positive control) for 72 h, showing coupled intensely shortened and curved dorsoventrally (C) with swollen areas (AS).

cause 93.75% mortality also at 96 h, reaching 100% lethality at 120 h with integumentary changes and separation of worms (Fig. 3E). According to the results of the statistical analysis the EC_{50} in 24 h was to concentration of 50 μM, as experimentally observed. Finally, the worms exposed to PZQ presented reduced motor activities, with 81.25% of the worms with score 0 at the interval of 24 h. After 48 and 72 h the percentages corresponded to 93.75 and 100% lethality, respectively, with integumentary alterations but without the separation of worms (Fig. 3F).

It was observed that changes in the viability (motility and death) of *S. mansoni* worms exposed to potassium usnate were dose- and time-dependent. Levi-Schaffer et al. (1984) identified that changes in the viability of *S. mansoni* worms are associated with deleterious effects on the cholinergic nervous system of the parasite, mainly affecting the catalytic activity of acetylcholinesterase after exposure to phosphonium salts and phosphoranes. In this way, it is observed that the reduction in the viability of *S. mansoni* worms exposed to potassium usnate occurred gradually at sublethal concentrations 50, 25 and 12.5 μM for all observation time intervals, which could suggest that this deficit may be directly associated with the neurological alterations caused by potassium usnate on the parasites. In a study that involved incubating *S. mansoni* worms with curcumin at concentrations of 50 and 100 μM, Magalhães et al. (2009) observed a reduction in viability and separation of individual male and female worms after 24 h of incubation, with 100% mortality and integumentary alterations only at 100 μM, while continuous exposure for 120 h to 50 μM was necessary to achieve the same percentage of mortality. Meanwhile, 100% mortality of worms was only reached at the 200 μM concentration of usnic acid after 120 h of exposure (Salloum et al., 2012). Thus, we observed that the modification of the molecular structure of usnic acid into potassium usnate shows enhanced activity against coupled adult *S. mansoni* worms at concentrations and intervals of five times less, possibly due to the presence of the K^+ ion in its structure (Fig. 1). In addition, our research group explored of usnic acid, and potassium usnate obtained from usnic acid, in control schistosomiasis through teratogenic and toxic effects on embryonic stages (blastula, gastrula, trochophore and veliger) and adult *B. glabrata* (Martins et al., 2014; Araújo et al., 2018a,b,c,d). In these studies, comparing the inviability of the embryos and adult *B. glabrata* exposed to the usnic acid and its derivative potassium usnate, it was demonstrated that mollusks (embryos and adult *B. glabrata*) showed greater susceptibility to potassium usnate, lethal concentrations 50 and 90% were lower with the exception of the blastula stage. Meanwhile, Yang et al. (2018) reported enhanced bioavailability and inhibition of invasion and metastasis in colorectal cancer in murine model after treatments with potassium usnate than when compared with usnic acid.

Regarding fecundity, *S. mansoni* females exposed to potassium usnate showed null capacity, since no eggs were observed at any concentration at the different observation intervals, differently from that observed for couples of the negative control, in which female worms performed oviposition at all observed intervals (Figs. 3A and 4). It is known that coupled worms remain paired in their host's blood system for the rest of their lives (decades), so drugs that even at sublethal concentrations alter the oviposition of *S. mansoni* worms are extremely important, considering that the disease symptoms of parasitized individuals are closely related to the process of retention of eggs in host tissues, mainly in the liver and intestine, and the host's immunopathological responses to them (Cioli et al., 2014). In the epidemiological aspect of the disease, the egg is fundamental to the biological cycle of the parasite, since inside each egg there is a single

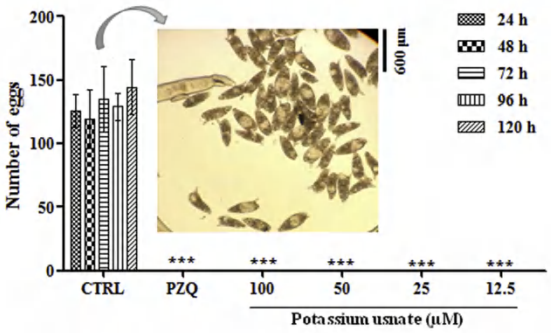

Fig. 4. *In vitro* effect of potassium usnate on egg production. Coupled adult *S. mansoni* worms were treated with potassium usnate at 12.5–100 µM concentrations during 120 h and the number of eggs was monitored using an inverted microscope. Values expressed as mean (± standard deviation).

miracidium that is responsible for generating up to 300,000 parasite cercariae (Brasil, 2014). Studies by Moraes et al. (2011) and Salloum et al. (2012) also demonstrated reduced oviposition of *S. mansoni* females exposed to piplatin and usnic acid, at concentrations of 9.5 and 100 µM respectively. However, for usnic acid, a complete reduction of oviposited eggs was observed after exposure for 120 h. However, from the first observation interval (24 h) at the 12.5 µM concentration potassium usnate showed a 100% reduction in oviposition capacity by *S. mansoni* females (Fig. 4).

Through the photomicrograph we observed progressive changes in the worms exposed to the potassium usnate at the different concentrations and time intervals (Fig. 3B–E) and PZQ (Fig. 3F), of which the following can be highlighted: swellings, bubbles, dorsoventral contraction, and spiral, short and curved worms. This study provides consistent evidence that potassium usnate is a promising schistosomicidal agent, acting on coupled *S. mansoni* worms at different time intervals and at low concentrations, while also altering important parasite parameters.

According with the Organization for Economic Cooperation and Development (OECD), technical specifications indicate that substances with an LD_{50} higher than 1000 mg/kg orally are considered safe or slightly toxic (OCDE, 2001). In terms of acute toxicity in a murine experimental model, potassium usnate at the concentration of 500 mg/kg did not present hematological, biochemical and histopathological alterations and even in concentration of 2000 mg/kg the LD_{50} was not determined because only caused as only 40% mortality (Araújo et al., 2019). These results highlighted the biosecurity of potassium usnate in the animal model. Thus, *in vivo* experimental studies are being conducted to evaluate the schistosomicidal potential of potassium usnate, as well it's mechanisms of action against *S. mansoni*. These results will be fundamental for futures clinical trials to be conducted. Furthermore, potassium usnate may be investigated as adjunct to the PZQ against etiological agents of schistosomiasis. In conclusion, our results indicate that potassium usnate represents a possible candidate for further study as a potential antischistosomal agent.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2019.107779.

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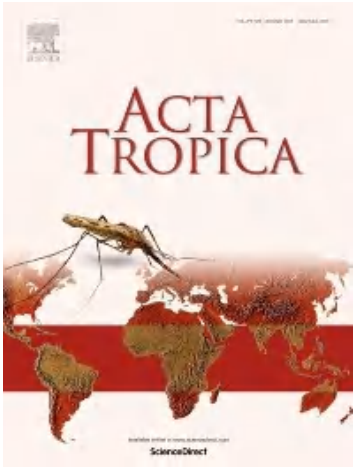
3.4 ARTIGO 4

USNIC ACID POTASSIUM SALT FROM *Cladonia substellata* (LICHEN): SYNTHESIS, CYTOTOXICITY AND *IN VITRO* ANTHELMINTIC ACTIVITY AND ULTRASTRUCTURAL ANALYSIS AGAINST ADULT WORMS OF *Schistosoma mansoni*



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Usnic acid potassium salt from *Cladonia substellata* (Lichen): Synthesis, cytotoxicity and *in vitro* anthelmintic activity and ultrastructural analysis against adult worms of *Schistosoma mansoni*

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ABSTRACT

We report for the first time the *in vitro* effect of Potassium Salt, derived from Usnic Acid (PS-UA), isolated from the lichen *Cladonia substellata* Vanio, on couples of *Schistosoma mansoni*. As schistosomicide parameters, we evaluated mortality, motility, cell viability of the worms and tegument changes by scanning electron microscopy (SEM). Exposure to a concentration of 100 μM caused 75% mortality after 3 h. After 6 h, changes in motility in concentrations of 50 and 25 μM are evidenced. After 12 h and 24h, the concentrations of 50 and 100 μM caused 6.25% and 87.5% and 50% and 100% mortality, respectively. PS-UA reduced the cell viability of the worms by 27.36% and 52.82% at concentrations 50 and 100 μM, respectively. Through SEM we observed progressive dose- and time-dependent, alterations such as swelling, blisters, dorsoventral contraction, erosion until disintegration of the tubercles in the tegument of male and female. PS-UA did not alter the viability of human peripheral blood mononuclear cells and showed high selectivity indices (IC_{50} > 200 μM). Our results indicate that PS-UA represents a possible candidate for a new anthelmintic drug in the control of schistosomiasis.

1. Introduction

Schistosomiasis is a potentially fatal infection caused by blood helminths *Schistosoma* spp. Schistosomiasis puts at risk around 800 million people distributed in 78 countries and territories of the tropical and subtropical regions of the world. The disease affects about 265 million people and accounts for 200 thousand deaths annually (World Health Organization, 2015a). *Schistosoma mansoni* is the most prevalent species found in the African continent, and is the only one found in Central and South America, including Brazil (Noya et al., 2015; World Health Organization, 2015a). The severe form of schistosomiasis mansoni is characterized by periportal fibrosis, intrahepatic veins obstructed by eggs, presinusoidal portal hypertension, splenomegaly, hemodynamic alteration, lipid abnormalities, and upper digestive bleeding (Tischendorf et al., 1996; Katz and Peixoto, 2000; Leite et al., 2013, 2015; Dias et al., 2013; Fonseca et al., 2014; Barbosa et al., 2016).

In the absence of a vaccine, biosecurity and lack of access to essential commodities and services, such as clean water and improved sanitation the strategy used to reduce the prevalence and incidence of schistosomiasis depends solely on the chemotherapy performed with Praziquantel (PZQ) (Webster et al., 2013; Diniz et al., 2014; Favre et al.,

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0001-706X/

2015; Utzinger et al., 2015). Estimates show that at least 206.4 million people needed preventive treatment for schistosomiasis in 2016, out of which only 89 million people were treated (World Health Organization, 2018). The enormous demand for its use is confirmed not only by the high prevalence of schistosomiasis but also by numerous cases of re-infection that are reported on an annual or semi annual basis (World Health Organization, 2015b; 2016; 2017). This scenario is responsible for the emergence of *Schistosoma* strains resistant or tolerant to PZQ and, as a consequence, to a future collapse in the treatment of schistosomiasis (Pica-Mattoccia et al., 2009; Vale et al., 2017). Thus, our group has explored the schistosomicidal potential of new molecules of natural, semi-synthetic or synthetic origin in the control and treatment of schistosomiasis (Bertão et al., 2012; Santos et al., 2014; Aires et al., 2014; Rocha-Filho et al., 2015; Silva et al., 2018).

Lichen or lichenized fungi are symbiotic organisms that have at least one fungus (mycobiont, heterotrophic) and a green algae or cyanobacteria (photobiont, autotrophic) that produce several secondary and bioactive metabolites of pharmacological importance (Yousuf et al., 2014; Calcott et al., 2018). Among the great diversity of lichens found in tropical and subtropical regions, *Cladonia substellata* Vanio (1887) is found in the Northeast Region of Brazil (Fig. 1). Among the secondary metabolites produced by this species, usnic acid is prominent, being the major metabolite and presenting pharmacological activities including antimicrobial, antioxidant, antiviral, anti-inflammatory, antitumor and antiparasitic effects (White et al., 2014; Araújo et al., 2015). A study by Salloum et al. (2012) reported that the acetone extract and the usnic acid obtained from *Usnea steineri* were able to cause 100% mortality against *S. mansoni* adult worms. Although usnic acid presents important biological activities, its low solubility represents a limiting factor. Thus, its modification as a potassium salt (PS-UA) is a strategy that can be employed to make it soluble without losing its biological potential, thus facilitating administration of the drug (Göke et al., 2018).

The objective of the present study was to evaluate the schistosomicidal potential, *in vitro*, of PS-UA, obtained from *Cladonia substellata* Vanio, through mortality, motility, cell viability of the worms and tegument alterations by scanning electron microscopy, against adult worms of *S. mansoni*, in addition to evaluating the cytotoxicity of PS-UA on human peripheral blood mononuclear cells (PBMC).

2. Material and methods

2.1. Samples of Cladonia substellata Vanio

C. substellata Vanio (1887) samples were collected in the Northeast Region of Brazil, in the municipality of Mamanguape (state of Paraíba, Brazil), 6°42′1.5″ S/35°8′3.3″ W (Fig. 1), in sandy soils in the summer period in the Southern Hemisphere (February 2015). A sample (voucher nº 77.474) was deposited in the herbarium Geraldo Mariz, Department of Botany of the Federal University of Pernambuco (UFPE), Recife/PE, Brazil.

2.2. Compounds

Praziquantel (purity ≥ 98%) was purchased from Sigma Chemical Co. (St. Louis, MO, EUA); Potassium hydroxide was purchased from Merck KGaA (Darmstadt, Germany). All other analytical or cell culture were of grade reagents and were purchased from Sigma-Aldrich (Brazil).

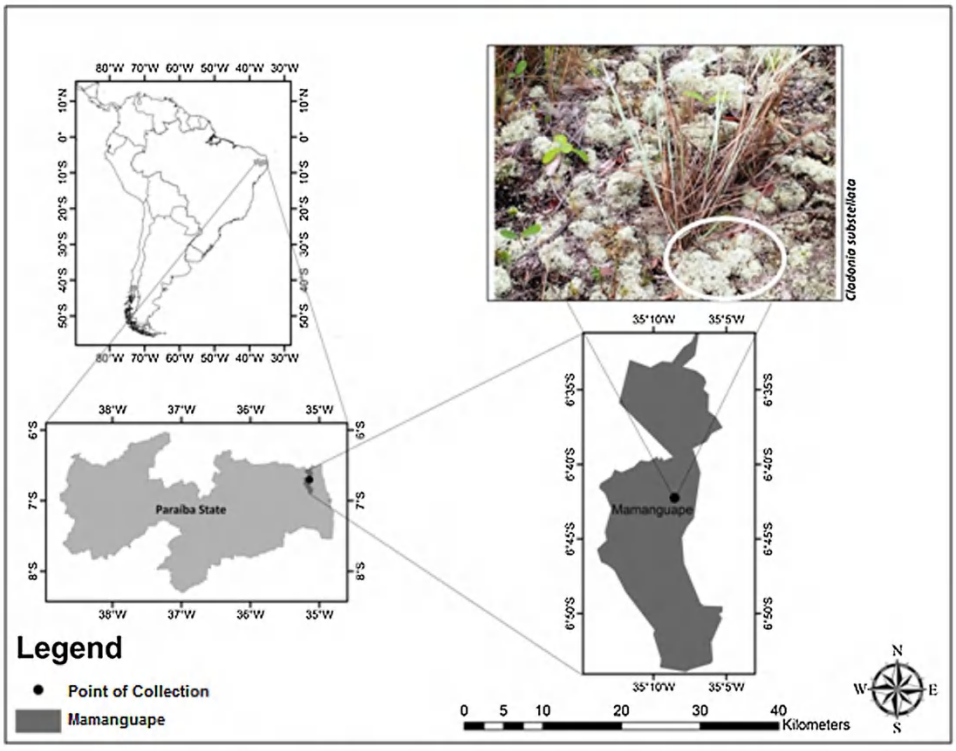

Fig. 1. Geographical location of the city of Mamanguape, (Paraíba, Brazil and South America). Indicating the point of collection of lichen *Cladonia substellata*.

2.3. Etheric extract preparation, isolation and purification of usnic acid

C. substellata samples (100 g) were cleaned, dried, and ground to a powder, which was subsequently subjected to successive extractions with diethyl ether (150 mL) in a Soxhlet apparatus at 40 °C until exhaustion of the thallus (6x). After each extraction, organic extracts were kept at 4 °C (24 h) and filtered. Then, the extracts were dried in a rotary evaporator coupled to a water bath at 37 °C. The usnic acid was isolated and purified as previously described (Araújo et al., 2018a). Briefly, 230 mg of the dry extract was fractionated on a silica gel column (70–230 mesh), eluted in the solvent chloroform hexane system (80:20 v/v), and evaporated until dry. The fractions obtained were monitored by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Those samples that showed only one band were combined. The fractionation and monitoring processes were repeated until highly pure usnic acid (> 98%) was obtained, and the molecular structure analyzed by the spectra of proton nuclear magnetic resonance (^{1}H NMR) and Carbon (^{13}C NMR) obtained at 400 MHz in $CDCl_3$ (Varian UNITY spectrometer), while infrared spectroscopy (IR) analyses were performed in a Bruker Fourier spectrometer (model IFS 66) with KBr disks.

2.4. Synthesis of PS-UA

To obtaln PS-UA, usnic acid was placed in a glass beaker and milli-Q water was added at 40 °C, then 5% potassium hydroxide was added until the solubilization of the sample at pH 11. Finally, the sample was lyophilized (Araújo et al., 2018b). The structure of PS-UA molecule was confirmed by IR spectroscopy and ^{1}H NMR.

2.5. Ethical considerations, animals and infection

After approval of the Ethics Committee in Animal Experimentation of the Bioscience Center, UFPE (Proc. Nº 23076015163/2017-65), female *Swiss* mice, weighing 28 ± 2 g, were percutaneously infected (Olivier and Stirewalt, 1952), with about 120 cercariae of *S. mansoni* (BH strain), maintained at the Schistosomiasis Experimental Laboratory of the Keizo Asami Immunopathology Laboratory - LIKA/UFPE. Mice were obtained and kept in the LIKA vivarium, in a controlled environment (20 ± 2 °C, 12 h daylight cycle) with free access to food (Labitum/Purina, São Paulo-SP) and water.

2.6. In vitro studies with S. mansoni

Sixty days after infection, the mice were euthanized by cervical dislocation and the worms were aseptically recovered by perfusion of the portal system and mesenteric veins with sterile saline (0.9% NaCl w/v) (Smithers and Terry, 1965). Only intact worm couples were immediately transferred to an RPMI 1640 medium supplemented with 20 mM HEPES, 100 μg/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum, being rinsed four times with this medium. Then, the worms were distributed in 24-well culture plates with 2 mL of this medium (two worm couples per well), and incubated at 37 °C in a humid atmosphere containing 5% CO_2. After two hours of exposure, to enable the adaptation of the worms, PS-UA (solubilized in RPMI 1640 culture medium) was added to a final concentration of 12.5, 25, 50 or 100 μM. The control worms were assayed in RPMI 1640 medium as a negative control group and in 10 μM PZQ as a positive control group. Two independent experiments (16 pairs of worms per concentration), were performed in quadruplicate (El-Beshbishi et al., 2015).

2.7. Antischistosomal evaluation criteria

2.7.1. Motility and survival

An inverted microscope (Leica Microsystems, DM IL Wetzlar, Germany) was used to evaluate the motility and survival of worm couples monitored after 3, 6, 12 and 24 h of exposure. Motility and survival of worms were assessed according to the criteria and scored in a viability scale of 3-0 as proposed by Silva et al. (2018), being: score 3, worms that present typical movements, exhibiting peristalsis of the internal organs, suckers in movement, adhering to the bottom or sides of the culture plate; typical descriptions of worms of the negative control; score 2, reduced movements throughout the body, peristalsis of internal organs and suckers; score 1, movements only at the extremities or at only one of the extremities (anterior and/or posterior regions), with absence of peristalsis of the internal organs and not adhered suckers; score 0, complete absence of motions and tegument with or without changes in coloration. The treatment was considered lethal when it was not possible to observe parasite movements for up to 2 min.

2.7.2. Cell viability assay of couples of S. mansoni worms

The cell viability of worm couples *S. mansoni* after exposure to PS-UA for 24 h was determined by cytotoxicity assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), according to conditions previously reported by Aires et al. (2014). Briefly, two worm couples were placed in individual wells on 96-well plates containing 100 μL of MTT (5 mg/mL in phosphate-buffered saline - PBS) and incubated at 37 °C for 30 min. Thereafter, the MTT solution was replaced by 200 μL dimethyl suifoxide (DMSO), with the purpose of dissolving the purple formazan crystals, and the optical density measured at 550 nm, in a microplate reader (M680, Bio-Rad Laboratories, Inc.). Again, as control groups, two worm couples were incubated in RPMI 1640 (negative control), and exposed to 10 μM PZQ (positive control) for the same time intervals and experimental conditions. Two independent experiments were performed in quadruplicate.

2.7.3. Scanning electron microscopy

For ultrastructural analysis of PS-UA activity against adult *S. mansoni* worms, scanning electron microscopy (SEM) was used. Worm couples incubated in 50 or 100 μM PS-UA for 3, 6, 12 or 24 h were sampled and fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in a 0.1 M sodium cacodylate buffer (pH 7.2) for 12 h at room temperature. Thereafter, samples were washed in the same buffer and post-fixed with 1% (w/v) OsO_4 in a 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature. Specimens were then dehydrated with increasing concentrations of ethanol (30, 50, 70, 90 and 100%) for 10 min each step. After dehydration, the critical point for the substitution of ethanol with carbon dioxide was obtained, drying the material and mouting it on metallic stubs using double-sided carbon tape. Metallization was then performed by covering the material with a thin layer of gold for visualization and analysis on the scanning electron microscope JEOL JSM-5600 LV.

2.8. Cytotoxicity assay using human normal cells

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood from healthy individuals (n = 5). The assay was performed according to the methodology described by Albuquerque et al. (2014). Cells were only used when viability was > 98%. PBMC (10^6 cells/mL) were incubated for a period of 72 h with PS-UA at concentrations ranging from 1.56 to 200 μM. After the exposure period, MTT was added at 5.0 mg/mL. After 4 h, the MTT metabolism product was dissolved in DMSO, the absorbance measurement was performed at 450 nm. Negative and positive controls corresponded to the cells not treated with PA-UA or treated with etoposide phosphate (0.625–10 mg/mL), respectively. The assays were performed in quadruplicate in three independent experiments. All donors signed an informed consent form and the study was approved by Resolution 466/12 of the National Health Council (CAAE) 62919816.2.0000.5208.

2.9. Statistical analysis

Numerical data were analyzed with Graphpad Prism 5 software (GraphPad Software, Inc., La Jolla - CA, USA) and are expressed as mean ± standard deviation (SD). Statistical differences were determined by using one-way analysis of variance (ANOVA) in conjunction with Tukey's test for post-hoc multiple comparisons. The significant differences were taken as $p < 0.05$.

3. Results

3.1. Chemical analysis

The TLC analysis demonstrated the presence of a single band with crystals of usnic acid with a retention factor (Rf) 0.84 compatible with the band of the standard of the acid Rf 0.84. This result was confirmed by HPLC with Rf (20.49) of the standard single acid with the Rf (20.46) of our samples. The following spectra of ^{1}H NMR, ^{13}C NMR and IR were obtained: ^{1}H NMR: (400 MHz, Acetone-d_6) δ_H (H; *mult.;* int.): 1.76 (3H; s, CH_3-13), 2.15 (3H; s, CH_3-16), 2.66 (3H; s, CH_3-15), 2.68 (3H; s, CH3-18), 5.98 (1H; s, C-4-H), 11.02 (1H; s, C-10−OH), 13.31 (1H; s, C-8−OH), 18.85 (1H; s, C-3−OH). ^{13}C NMR: (400 MHz, Acetone-d_6) δ_C: (C; *mult.;* int.): C-1: 198.05; C-2: 191.70; C-3: 157.50; C-4: 98.32; C-5: 101.53; C-6: 76.99; C-7: 155.20; C-8: 163.89; C-9: 103.94; C-10: 179.38; C-11: 109.34; C-12: 59.06; C-13: 27.87; C-14: 200.30; C-15: 32.10; C-16: 7.52; C-17: 201.76; C-18: 31.25. IR (KBr): 3090 (ν C–H Ar); 3005 (ν_{as} CH_3); 2925 (ν CH_3); 1695 (ν C = 0); 1635; 1550 (ν C=C Ar); 1446 (δ_{as} CH_3); 1385 (δ_s CH_3) cm^{-1} (Fig. 2A).

Subsequently, the synthesis of PS-UA was carried out and confirmed by ^{1}H NMR and IR, from which the following data were obtained: ^{1}H NMR (400 MH_z, acetone- d_6) δ-h:1.62 (3H, s, CH_3-13); 1.98 (3H, s, CH_3-16); 2.61 (3H, s, CH_3-15); 2.82 (3H, s, CH_3-18); 5.53 (1H, s, C-4-H); 13.44 (1H, s, C-10−OH); 14.30 (1H, s, C-3−OH). IR (KBr): 3455 (ν C−OH); 3096 (ν C–H Ar); 2989 (ν_{as} CH_3); 2929 (ν_s CH_3); 1697 (ν C = 0); 1638; 1572 (ν C=C Ar); 1446 (δ_{as} CH_3); 1380 (δ_s CH_3); 1150-1070 (ν C−0−C) cm^{-1} (Fig. 2B).

3.2. PS-UA alters the motility and cellular viability of adult S. mansoni

Table 1 shows the mortality kinetics results of couples of adult *S. mansoni* worms exposed to PS-UA at the intervals of 3, 6, 12, and 24 h. After, 3 hs of exposure, 75% of the worms exposed to the concentration of 100 μM did not present any movement along the body, showing also darkened tegument (score 0) and writhing that made them smaller and coiled. After 6 h, 75% of the worms exposed to a concentration of 50 μM presented movements only at one or both extremities, with an absence of peristalsis of the internal organs and were not adhered to the culture plate (score 1). Also, in this interval, at the concentration of 25 μM, 75% of the exposed worms presented reduced movement throughout the body and peristalsis of the internal organs (score 2). After 12 h, mortality at the 50 and 100 μM concentrations were 6.25 and 87.5%, respectively. While after 24 h, those values were 50 and 100%, respectively. Moreover, at 12 h and 24 h, 93.75% worms exposed to 25 μM concentration showed reduced motility. At all time points worms exposed to the concentration of 12.5 μM showed no alteration in motility. This behavior was similar to the worms of the negative control group, which showed typical movements, exhibiting peristalsis of the internal organs, suckers in movement or adhering to the bottom or sides of the culture plate (score 3). In contrast, all worms exposed to 10 μM of PZQ (positive control group) were contracted, with blackened tegument and their motor activities were significantly reduced in the first 3 h, all presented signs of score 1. After 12 and 24 h of exposure, PZQ caused 62.5 and 81.25% mortality, respectively.

PS-UA was able to significantly reduce mitochondrial viability, and consequently cell viability, from the reduction of formation of formazan crystals. PS-UA, reduced the cell viability to 22.32%, 27.36% and 52.82% when the worm couples were incubated at concentrations of 25, 50 and 100 μM respectively, when compared to the negative control group. In addition, the concentration of 100 μM of PS-UA caused greater cell non-viability in couples of *S. mansoni* adult worms when compared to the positive control, worms exposed to PZQ ($p < 0.05$) (Fig. 3).

3.3. Ultrastructural analysis of PS-UA-induced surface damage in S. mansoni

For the ultrastructural analysis, worm couples were incubated in PS-UA at concentrations of 50 and 100 μM for 3, 6, 12 and 24 h, as throughout this interval these concentrations resulted in alterations in motility and caused death.

Worms incubated for 24 h in a supplemented RPMI 1640 medium showed intact surface structure and topography (Fig. 4A–D). In Fig. 4A we see couples *S. mansoni* worms with the female in the male gynecological channel. Fig. 4B shows the dorsal middle region of the male worm, evidencing the presence of tubercles with spines, in addition to ciliated papillae, dome-shaped papillae, folds between tubercles and spines in the tubercles. The anterior portion of male worms (Fig. 4C) and females (Fig. 4D) is characterized by an oral sucker or acetabulum (OS) and a ventral sucker or acetabulum (VS).

After 3 h of exposure to PS-UA at a concentration of 50 μM, bent adult worms were observed (Fig. 5B), while in males (Fig. 5A and D) it was possible to observe an extensive area of the tegument with tubercle deformation, areas with swellings, cracks and presence of bubbles, and changes in the anterior region of the female (Fig. 5C). After 6 h, changes

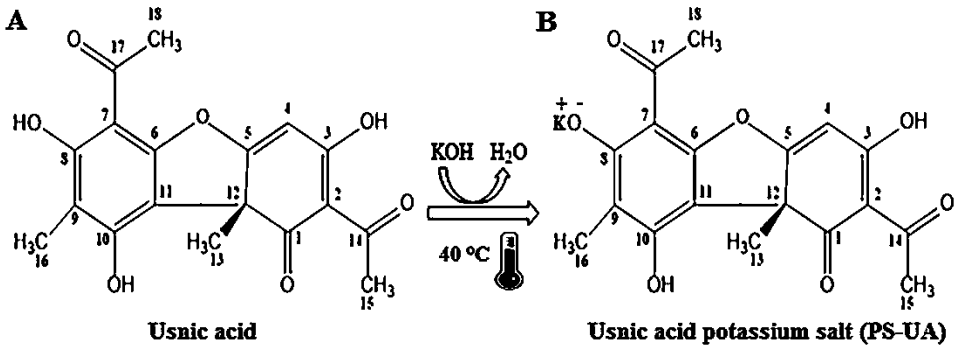

Fig. 2. Synthesis of usnic acid potassium salt - PS-UA.

Table 1
Motility score of control worms, treated with Praziquantel (PQZ -10 μM) and with PS-UA (100 - 12.5 μM) after 3, 6, 12 and 24 h of incubation.

Groups	Mean ± standard deviation (SD) and percent of worms (%) in motility scores after incubation																			
	3 h				6 h				12 h				24 h							
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3				
Ctrl Negat.				16 ± 0.0 (100%)				16 ± 0.0 (100%)				16 ± 0.0 (100%)				16 ± 0.0 (100%)		
Ctrl PZQ 10 μM		16 ± 0.0 (100%)			2 ± 1.41 (12.5%)	14 ± 1.41 (87.5%)			10 ± 2.82 (62.5%)	6 ± 2.82 (37.5%)			13 ± 1.41 (81.25%)	6 ± 1.41 (18.75%)						
PS-UA																				
100 μM	12 ± 4.24 (75%)	4 ± 1.41 (25%)			12 ± 4.24 (75%)	4 ± 1.41 (25%)			14 ± 2.82 (87.5%)	2 ± 2.82 (12.5%)			16 ± 0.0 (100%)							
50 μM		6 ± 1.41 (37.5%)	8 ± 2.82 (50%)	2 ± 0.0 (12.5%)		12 ± 2.82 (75%)	4 ± 1.41 (25%)		1 ± 1.41 (6.25%)	12 ± 2.82 (75%)	3 ± 1.41 (18.75%)		8 ± 2.82 (50%)	8 ± 2.82 (50%)						
25 μM			2 ± 1.41 (12.5%)	14 ± 5.65 (87.5%)			12 ± 2.82 (75%)	4 ± 1.41 (25%)			15 ± 1.41 (93.75%)	1 ± 1.41 (6.25%)			15 ± 1.41 (93.75%)	1 ± 1.41 (6.25%)				
12.5μM				16 ± 0.0 (100%)				16 ± 0.0 (100%)				16 ± 0.0 (100%)				16 ± 0.0 (100%)				

Note: percentage values of 32 worms (16 pairs of worms per concentration) per group. Two independent experiments.
Score 3 = present typical movements, exhibiting peristalsis of the internal organs, suckers in movement, adhering to the bottom or sides of the culture plate.
Score 2 = present reduced movements throughout the body, peristalsis of internal organs and suckers.
Score 1 = present movements only at the extremities or at only one of the extremities (anterior and/or posterior regions), with absence of peristalsis of the internal organs and not adhered suckers.
Score 0 = complete absence of motions and integument with or without changes in coloration.

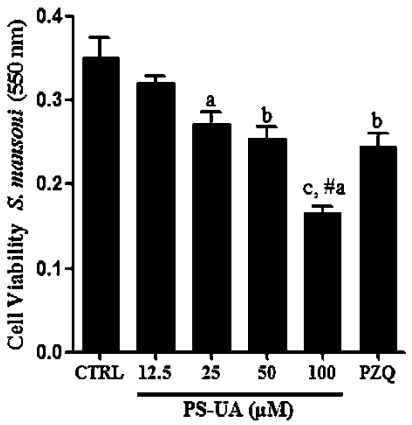

Fig. 3. *In vitro* effects of PS-UA (12.5, 25, 50 e 100 μM) on cell viability of couples of *S. mansoni* adult worms. Positive controls worms were treated with Praziquantel (PQZ, 10 μM). The viability was expressed as mean ± standard deviation (SD) of the absorbance values from four experiments. ^{a} = $P < 0.05$, ^{b} = $P < 0.01$ e ^{c} = $P < 0.001$ compared to the control (CTRL). $^{\#a}$ = $P < 0.05$ compared to positive control (PZQ).

were observed along the dorsal region of males (Fig. 5E–G) and contraction in the anterior region of the female with ventral sucker invagination (Fig. 5H). At 12 h of exposure, changes were observed in the anterior and dorsal regions of the male with loss of spicules and erosion of tubercles (Fig. 5I and J), while in the female it was possible to observe subtegumentar tissue (Fig. 5 K and L). At 24 h the changes show a strongly curved pair (Fig. 5N), with tegument erosion, loss of tubercles, spicules, bubbles and extensive area of exposed subtegument tissue in males (Fig. 5 O and P), while in the female there are peeling and holes (Fig. 5M).

After 3 h of exposure to PS-UA at the concentration of 100 μM, intense swelling (Fig. 6A) was observed in the male in the anterior region, cracks in the ventral suction cup and tegument with tubercle displacement (Fig. 6B–D). At 6 h, the anterior region of the male became wrinkled with furrows and had a fibrous appearance, extensive tegument erosion with exposure of subtegumentar tissue and intense presence of blisters (Fig. 6E–H). After 12 h of exposure, a coiled pair (Fig. 6J) was observed, with severe damage to the lateral dorsal region of the male tegument (Fig. 6I), in the female, grooves and exposure of the muscular layer were observed (Fig. 6 K and L). After 24 h of exposure, several changes became more intense and evident in the worms. In the female, strong grooves with aspects of coalescing folds and deep holes (Fig. 6 M and N) were observed. The males showed total dlsintegration of the tegument with exposure of the subtegument tlssue (Fig. 6O and P).

The effect of PZQ on *S. mansoni* worm pair shortly after 3 h of exposure left them curved and short due to contraction of the longitudinal muscles with many blisters (Fig. 7A). The effects after 6, 12 and 24 h (Fig. 7B–D) of exposure included numerous bubbles with swollen teguments, loss of spicules, juxtaposed tubercles and appearance of holes in the tegument.

3.4. Effect of PS-UA on human cells

Regarding PS-UA cytotoxicity on human peripheral blood mononuclear cells (PBMC), no toxicity was observed for concentrations that had a schistosomicidal effect (IC_{50} > 200 μM).

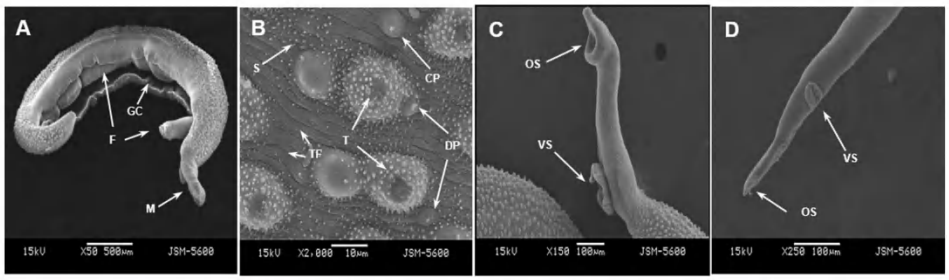

Fig. 4. A–D. Electromicrographs of *S. mansoni* adult couples from the negative control group (RPMI 1640 medium). (A, 50x), intact surface structure and normal morphological topography of the couple (F = female and M = male) with female in the male gynecophoric canal (GC). (B, 2,000x) enlarged view of dorsal male region it is possible to observe tubercles (T) with spines, parallel folds between tubercles (TF), spines (S), ciliated papillae (CP), and dome-shaped papillae (DP). In anterior region of male (C, 150x) and female (D, 250x) worms ventral sucker (VS) and oral sucker (OS).

4. Discussion

This is the first report of the schistosomicidal activity of PS-UA isolated from *C. substellata* against couples of adult *S. mansoni* worms. Our results show that PS-UA was able to cause changes in motility and tegument, mortality and reduction in cell viability of *S. mansoni* worms, which were more pronounced than those of PZQ according to the same parameters used here. Research on new drugs for the treatment of schistosomiasis continues to be a major challenge because, in addition to the schistosomicidal effect, these drugs must be biosecure, presenting tolerable limits of toxicity and cellular selectivity (Campelo et al., 2018). In the present study, according to the concentrations employed and the schistosomicidal effect, PS-UA was shown to be biosecure since it presented an $IC_{50} > 200\,\mu M$ for PBMC cells, a concentration double that which presented a schistosomicidal effect (100 µM). Despite their slow growth (Calcott et al., 2018), lichens and their derivatives, such as usnic acid, are important resources for the pharmaceutical industries (Rafanelli et al., 1995; Rancan et al., 2002; Nybakken and Julkunen-Tiitto (2006). Increased yields of lichenic compounds can be easily achieved with biotechnological techniques, including culture of symbionts or tissues and cellular immobilization (Garcöa-Junceda and Vicente, 1991; Pereira et al., 1995; Blanch et al., 2001). With the use of the latter technique, it is possible to obtain significant percentages of secondary metabolites from *C. substellata*, such as usnic acid as reported by Martins et al. (2017). In addition, by chemo-enzymatic assays, single-doses can be obtained synthetically from trihydroxyacetophenone, which is already commercially available (Hawranik et al., 2009). Thus with the use of biotechnology, PS-UA can be obtained in significant quantity for commercial use.

PS-UA, acid-base reaction product of the isolated usnic acid from *Cladonia substellata*, is characterized by the presence of benzene rings (phenolic character), ketone groups and a furan ring joining the benzene rings, with emphasis on the K^+ radical located on carbon 8. With respect to the schistosomicidal activity of usnic acid, Salloum et al. (2012) reported that 100% mortality of worms was only reached at the 200 µM concentration after 120 h of exposure, with changes in the tegument (appearance of bubbles and peeling). In our study, the concentration of 100 µM of PS-UA caused 100% mortality after 24 h of exposure. In addition, PS-UA caused a lethal effect on adult snails and on the different embryonic stages of *Biomphalaria glabrata,* that transmit schistosomiasis, at lower concentrations than that of usnic acid (Martins et al., 2014; Araújo et al., 2018a, 2018b, 2018c, 2018d). Therefore, we believe that the potentiality of the schistosomicidal activity of PS-UA is attributed to the K^+ radical, since the K^+ present in the structure of the molecule gives PS-UA hydrophilic characteristics, thus increasing its bioavailability and its biological and pharmacological effects (Göke et al., 2018).

Changes in motility and mortality of *S. mansoni* couples caused by PS-UA varied in a time and dose-dependent manner. These *in vitro* schistosomicidal effects have also been reported in studies by Moraes et al. (2011); Oliveira et al. (2012) and Silva et al. (2018) when evaluating piplatin (*Piper tuberculatum*), *Baccharis trimera* (less) DC, essential oil and benzodioxole derivatives, respectively. It is known that changes in motility that may result in the death of *S. mansoni* are associated with changes in neurotransmitters and/or neuromodulators such as serotonin, dopamine, acetylcholine, epinephrine, neuropeptides, glutamate and acetylcholinesterase (Sangster et al., 2005; Noël, 2008; Marks and Maule, 2010; Taman and Ribeiro, 2011).

The *S. mansoni* tegument is an important target in the development of new schistosomicidal drugs, since it makes first contact of the parasite with the drugs, besides being responsible for vital functions, among them protection against attack from the immune system of the host, nutrient absorption, lipid and cholesterol metabolism, and in the synthesis of some proteins (Skelly et al., 2014; Xavier et al.,2014; Sotillo et al., 2015; Silva et al., 2018). Thus, we aimed to evaluate the schistosomicidal effect of PS-UA on tegument changes, using scanning electron microscopy (SEM).

Other phenolic molecules of natural origin, such as plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), β-lapachone (3,4-dihydro-2, 2-dimethyl-2H-naphthol [1,2-b] pyran-5,6-dione) and artemisinin–naphthoquine phosphate were able to cause deep tegument alterations characterized by peeling of the tegument, formation and rupture of bubbles, appearance of holes, swelling, loss of spicules, dorsoventral contraction, lesion and disintegration of tubercles, exposure of the basal lamina and destruction of the tegument (Lorsuwannarat et al., 2013; Aires et al., 2014; El-Beshbishi et al.,2015). These changes are similar to those observed in our study. According to Fig. 5(J, L and O) and Fig. 6 (E, L, O, P), PS-UA caused extensive and severe tegument changes in *S. mansoni* worms. Thus, our hypothesis is that in an experimental model *in vivo* these tegument changes may favor the exposure of antigens on the surface of the worms, signaling the immune response of the intermediate host and thereby complementing the schistosomicidal action of PS-UA.

Although there has been no study on the action mechanism of PS-UA, there have been reports of possible pathways of action associated with the elements that make up its molecular structure. Methyl and cationic groups are related to the absorption of the molecule through the cell membranes by its facility in causing changes in cellular permeability as described by Roberts and Costello, (2003). Once inside the cell, the heterocyclic part together with the phenolic OH interact, inhibiting energy metabolism and acting in the decoupling of the electron transport chains. This hinders oxidative phosphorylation, thus causing hemostatic alterations in the basal gradient of protons inside the mitochondria and consequently cell death, as described by Joseph et al.

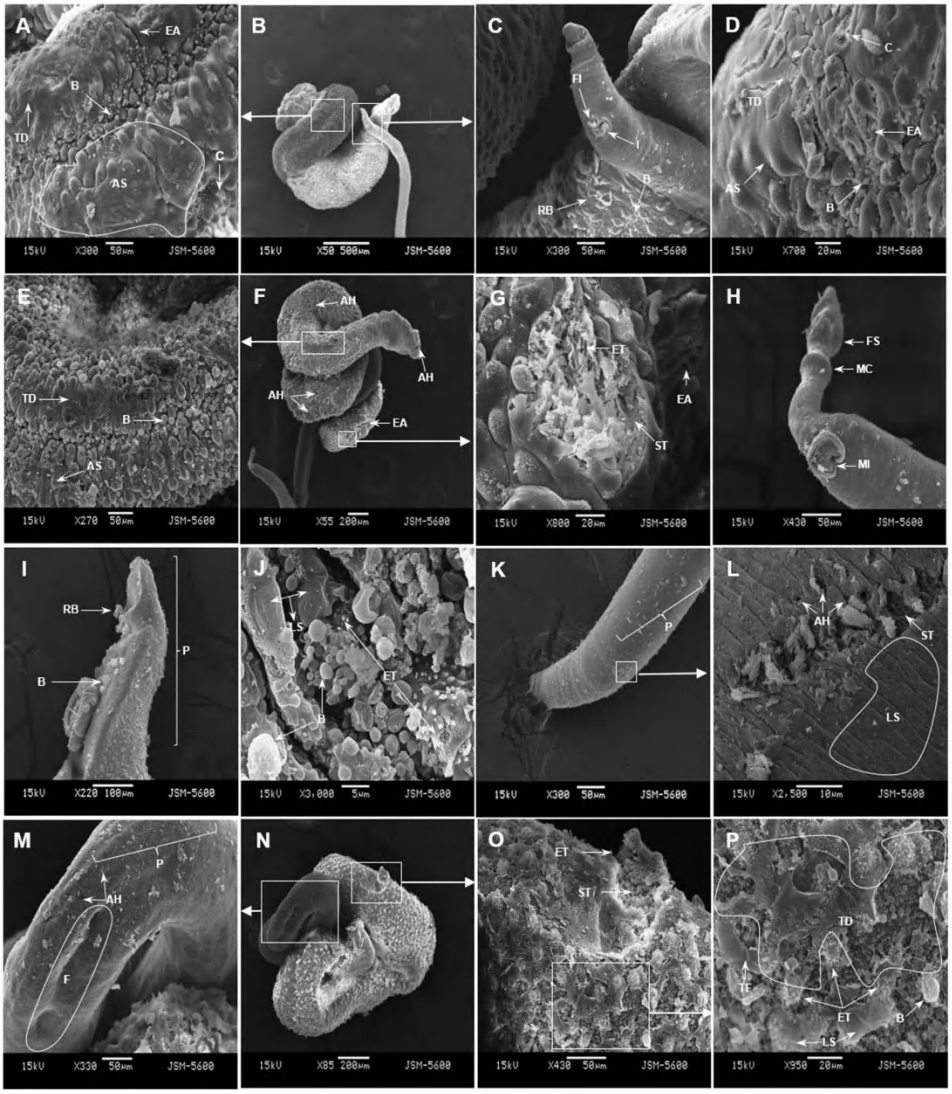

Fig. 5. A–P. Electromicrographs of *S. mansoni* adult couples exposed by 3 h to 50 μM PS-UA. After 3 h of exposure (A–D). In B (50x) couples were curved and in enlarged view (A, 300x), it is possible to observe extensive areas of the tegument with tubercles deformation (TD), swelling areas (AS), erosion (EA), cracks (C) and blisters (B) in the dorsal region of male worm, these changes are more visible after magnification (D, 700x). In C (300x) to observe focal lesion (FI) above ventral sucker of female worm, with a slight invagination (I) of ventral sucker and in proximal anterior region of male worm, blisters (B) presence, some of them ruptured (RB), distributed between the ciliated papillae. In the interval of 6 h (E–H), we observed a coiled couple (F, 55x), with emphasis on tegument lesions along the dorsal region of male worms with appearance of holes (AH) and erosion areas (EA). In E (270x), enlargement of anterior dorsal region of male worms, it is possible to identify deformations areas (TD) and swelling tubercles (AS), besides many bubbles (B). In G (800x), greater enlargement of posterior dorsal region of F, a large lesion in the tegument with erosion areas (EA) in the tegument and tubercles (ET) with subtegumentar tissue (ST) exposure. Female worm (H, 430x) showing anterior region with focal swelling (FS) and strong muscular contraction (MC) with mild invagination (MI) of ventral sucker. After 12 h of exposure (I–L), the picture I (220x) can seen bubbles (B) with some of them ruptured (RB) and peeling (P) in anterior region of male worm, being in *J* (3.000x) evidenced the dorsal region with bubbles (B) and loss of spicules (LS) and eruptions tubercles (ET). In K (300x) and L (2,500x) female worm with lesion on the tegument surface with peeling (P) and exposure of muscle tissue (ST), loss of spicules (LS) and appearance of holes (AH) respectively. In 24 h (M–P), N (85x) shows a strongly bent couple, focusing on extensive tegument destruction (TD) with erosion tubercles (ET), loss spicules (LS), parallel folds (TF), muscle tissue exposure (ST), as well as bubbles (B) in male worm, detailed in O (430x) and P (950x). In M (330x) we observed appearance of holes (AH), peeling (P) and furrows (F) in the female worm tegument.

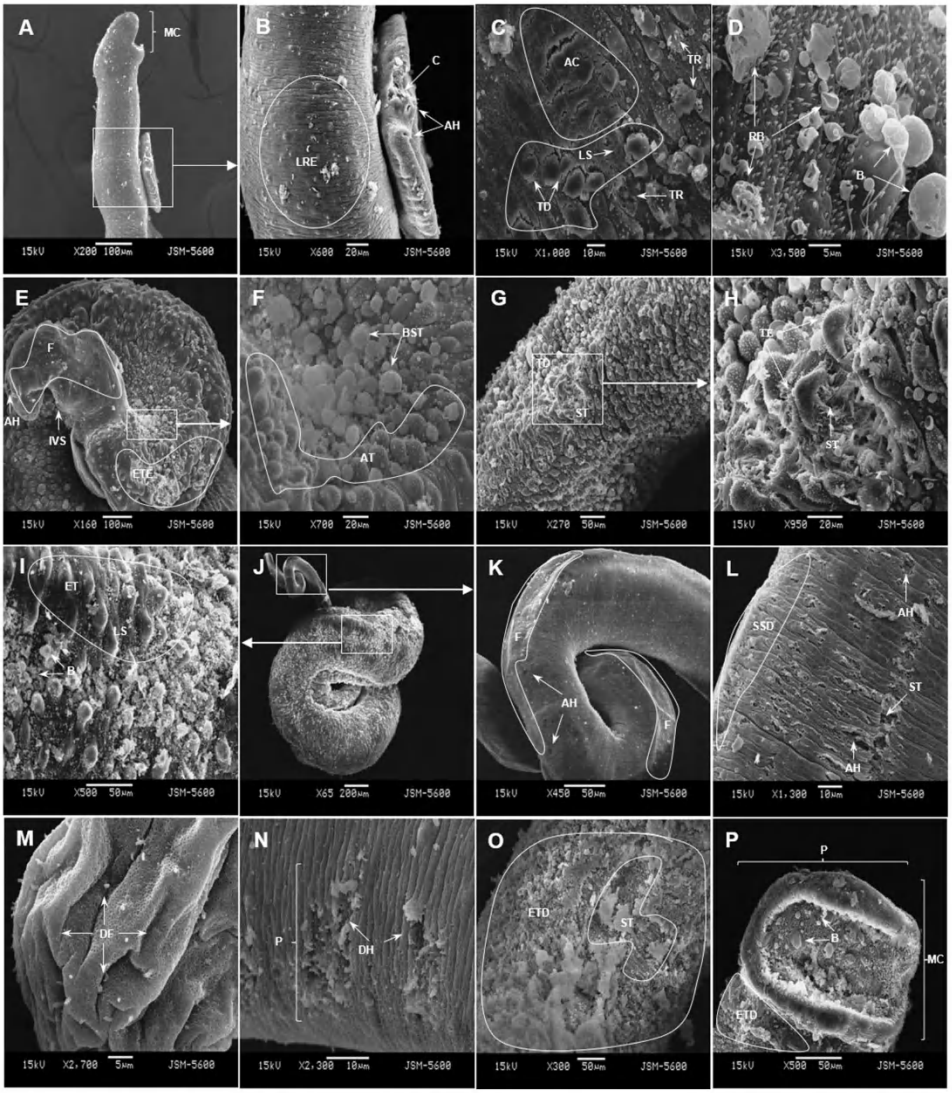

Fig. 6. A–P. Electromicrographs of *S. mansoni* adult couples exposed to 100 μM PS-UA. After 3 h of exposure (A–D). A (200x) and B (600x) showing anterior region of male worm evidencing retracted oral sucker (MC), lateral region edema (LRE), appearance of holes (AH) and cracks (C) around the ventral sucker. In C (1000x) area with cracks (AC), tubercles displacement (TD), loss of spicules (LS) and tubercles rupture (TR). In D (3,500x) presence of bubbles (B) with many ruptured (RB). After 6 h of exposure (E–H), in E (160x) evidence in male worm, extensive tegument erosion (ETE) in lateral dorsal region and anterior invagination of ventral sucker (IVS), furrows (F) and presence of holes (AH). In F (700x) we observe agglomerates of tubercles (AT) and bubble formation on the surface of the tegument (BST). In G (270x) tubercles displacement (TD), with exposure of subtegumental tissue (ST) evidenced in H (950x). After 12 h of exposure (I–L) J (65x) shows pair strongly curled. In I (500x) severe damage to lateral dorsal portion of tegument characterized by tubercles edema (ET), bubbles (B) and loss of spicules (LS) is observed. In K (450x) furrows (F) and appearance of holes (AH) along the female worm, while in L (1,300x) it highlights damaged sensorial structures (SSD) and presence of holes (AH) with different levels of severity, some with exposure of subtegumental tissue (ST). After 24 h of exposure (M–P), M (2,700x) shows the anterior region of female worm with deep furrows (DF). In N (2,300x) deep holes (DH) with peeling (P) tegument. In O (300x) extensive tegument destruction (ETD) is observed with submuscular tissue (ST) exposure in male worm. In P (1,500x) we observed the anterior region of the male worm with peeling (P), muscle contraction (MC), bubbles (B) and extensive tegument destruction (ETD).

(2009). It is important to report that the composition of the tegument of male and female worms of *S. mansoni* are differentiated. According to Hockley (1973), mitochondria are much more abundant in the tegument of male worms along the entire dorsal region compared to the tegument of female worms, and it is precisely in this region that the most severe and extensive tegument alterations were observed in male

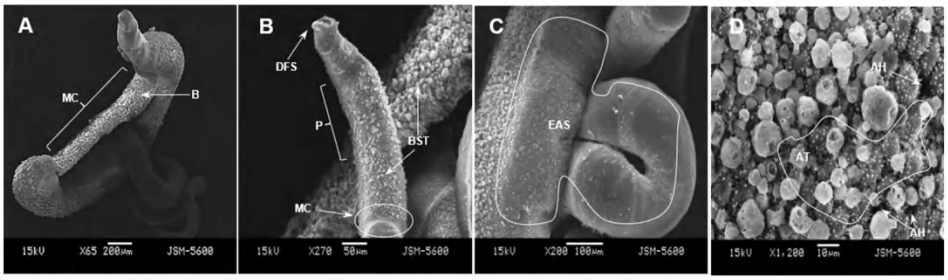

Fig. 7. A–D. Electromicrographs of *S. mansoni* adult couples exposed to PZQ (10 µM). After 3 h of exposure (A, 65x) visibly muscle contracted (MC), in median region the presence of bubbles (B) emerging around the tubercles of the male worm. After 6 h (B, 270x) the blisters became more numerous (BST) in male and female worms with damage to female sucker (DFS) and muscle contraction (MC) in the median region and peeling (P). At 12 h of exposure (C, 200x) the tegument was with extensive areas of swelling (EAS). After 24 h (D, 1,200x) in the dorsal region of male worms we observed agglomerated tubercles (AT) or juxtaposed and appearance of holes (AH).

worms, as can be seen in Fig. 5(D, E, J and O) and Fig. 6(E, G, O, P). Therefore, it is suggested that this is a possible pathway of the action mechanism of PS-UA, which acts on the mitochondria present in the tegument of male worms of *S. mansoni*, as described by Lorsuwannarat et al. (2013). While the tegument alterations caused by PZQ are characterized by bubbles, loss of tubers and spicules, and the presence of holes. According to Cioli et al. (2014), these changes are consequences of the influx of Ca^2+ ions that would initially cause muscle contractions in the worms, making them shortened and curved, with the presence of juxtaposed tubers, as can be seen in Fig. 7(A–D).

5. Conclusion

Taken together, our results indicate that PS-UA may be a good candidate in the search for new anthelmintic drug, and point to the potential of using PS-UA as an effective chemotherapeutic agent against the etiologic agent of schistosomiasis mansoni. However, further studies are needed in order to elucidate the pathophysiological effects of PS-UA in a murine experimental model, seeking to elucidate the mechanisms of toxicity of this molecule.

Conflict of interests

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Author contributions

H. D. A. Araújo, A. L. Aires, M. C. P. A. Albuquerque and V. L. M. Lima designed the study protocol. H. D. A. Araújo, A. L. Aires, M. C. P. A. Albuquerque, C. L. R. Soares, T. G. S. Brito, W. M. Nascimento, T. G. Silva, F. A. Brayner, L. C. Alves, M. C. B. Martins, N. H. Silva, and V. L. M. Lima carried out the assays and were involved in the analysis and interpretation of all data. H. D. A. Araújo, A. L. Aires, M. C. P. A. Albuquerque and V. L. M. Lima contributed to drafting the manuscript and/or critically revising the paper and intellectual content. All authors read and approved the final manuscript.

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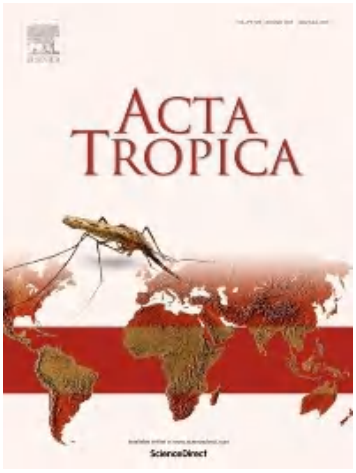
3.5 ARTIGO 5

IN VITRO ACTIVITY OF USNIC ACID POTASSIUM SALT AGAINST DIFFERENT DEVELOPMENTAL STAGES OF *Schistosoma mansoni*: AN ULTRASTRUCTURAL STUDY



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In vitro activity of usnic acid potassium salt against different developmental stages of *Schistosoma mansoni*: An ultrastructural study

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ABSTRACT

Currently, the control of schistosomiasis is based on a single drug, praziquantel, which is effective against all species of *Schistosoma* but only in the adult stage, presenting a schistosomicidal deficit at the other developmental stages of the parasites. Recently our research group has demonstrated that the potassium salt of usnic acid (PS-UA) presented schistosomicidal property against couples of adult worms of *S. mansoni*. Thus, the present study seeks to report for the first time the *in vitro* activity of PS-UA against different developmental stages of *S. mansoni* (schistosomules and young worms). As schistosomicide parameters, we evaluated motility, mortality, cell viability of the worms and tegument changes by scanning electron microscopy (SEM). After 3 h exposure, PS-UA was lethal to schistosomules at concentrations of 100 and 50 μM, whereas for concentrations 25 and 12.5 μM, 38 and 18% of mortality and 62 and 24% changes in motility, respectively, were reached. Yet for schistosomules, concentration of 25 μM caused 90 and 100% of death after 6 and 12 h, respectively. In the concentration of 12.5 μM at intervals of 12 and 24 h mortality was 68 and 100%, respectively. For young worms, after 3 h of exposure at concentrations of 200 and 100 μM caused 57 and 27% mortality, respectively. After 12 and 24 h, these concentrations caused mortality of 90 and 100% and 47 and 60% respectively. After 24 h, concentrations of 50 and 25 μM caused 80 and 30% change in motility, respectively. However, at the 12.5 μM concentration no change was observed. In addition, PS-UA reduced the cellular viability of young worms by 50.98% and 85.87% at concentrations of 100 and 200 μM, respectively. In both stages of worms and at different exposure intervals, PS-UA caused alterations such as: dorsoventral contraction, peeling, swelling, blisters, erosion, exposure of subtegumental tissue and disintegration of tegument. According to the results, changes in motility and mortality caused by PS-UA against schistosomules and young worms were concentration and time-dependents, also PS-UA even at low concentration, was able to cause profound ultrastructural changes in the integument of the worms. PS-UA is a promising candidate as prophylactic agent in the control of schistosomiasis mansoni.

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1. Introduction

Schistosomiasis is a neglected tropical infection caused by trematode helminths of the genus *Schistosoma* spp. (World Health Organization, 2019). That infection is responsible for major epidemiological impacts, since schistosomiasis is endemic in 78 countries and territories, affects approximately 250 million people, poses an imminent threat to an additional 800 million and exceeds 200,000 deaths each year (Van der Werf et al., 2003; Steinmann et al., 2006; Utzinger et al., 2011; World Health Organization, 2019).

Infection by *S. mansoni* occurs through contact with water sources contaminated with cercariae, an infective developmental phase for the definitive host, man. After penetration into the skin and/or mucous membranes, the cercaria becomes in schistosomule and remains at the point of penetration for up to 72 h, before reaching the blood or lymphatic circulation and reaching the lungs, where it remains until the 14th day of infection. Interestingly, the schistosomules present anatomico-physiological similarities from the beginning to the 14th day of infection (Hockley, 1973; Colley et al., 2014). After passing through the lungs, the schistosomule migrates to the portal-hepatic system where it completes its differentiation into a young worm, around the 21nd day after infection. Later, young worm mature into adult worm, male or female, at which time they form coupled pairs, mate and migrate to the mesenteric veins, where females begin oviposition around the 38th day of infection (Colley et al., 2014; Pereira et al., 2015).

Currently, Praziquantel (PZQ) is the only drug used in the treatment and control of schistosomiasis to minimize the incidence, prevalence, morbidity and severe clinical forms, since there is still no vaccine against schistosomiasis (Tischendorf et al., 1996; Van der Werf et al., 2003; Ming-Gang, 2005; Gouvras et al., 2013; Diniz et al., 2014). Although it is effective against adult worms of all species of *Schistosoma*, showing a high parasitic cure rate, PZQ does not present a prophylactic effect and at the recommended doses does not present activity against immature stages (schistosomules and young worms) (Xiao et al., 1985; Sabah et al., 1986; Oliveira et al., 2014) and there are reports of *S. mansoni* strains resistant and/or tolerant to PZQ (Melman et al., 2009; Pica-Mattoccia et al., 2009). This scenario is worrying, since in endemic regions infection and/or reinfection cases are common, where many patients may harbor worms in the adult stage and in different developmental stages concomitantly, and a few days after treatment with PZQ they present recurrent morbidity of the parasites that developed (Webster et al., 2015; Caffrey, 2015). Therefore, it is necessary to encourage research that seeks new schistosomicidal drugs in the treatment and prevention not only of adult worms, but also against different developmental stages of the parasite (Hines-Kay et al., 2012; Aires et al., 2014a; Pereira et al., 2015; Mossallam et al., 2015).

Usnic acid is a secondary metabolite found in several lichen species of the genus *Cladonia, Usnea, Lecanora, Ramalina, Parmelia* and *Evernia* (Ingólfsdóttir, 2002). Studies report several biological activities of usnic acid, among them: anticancer, antimicrobial, antiviral, anti-inflammatory, antioxidant, antiparasitic and molluscicidal (White et al., 2014; Araújo et al., 2015; Araújo et al., 2018a,b). However, the use of usnic acid is limited because of its hydrophobic properties. Thus, through an acid-base reaction between usnic acid and potassium, usnic acid is modified into a salt, usnic acid potassium salt (PS-UA), which presents high solubility in water, conferring to that molecule greater bioavailability and biological effects (Yang et al., 2018; Araújo et al., 2018c,d; Araújo et al., 2019a,b) (Fig. 1). PS-UA It's stabilized by the presence of benzene rings and hydroxyl, ketone groups and a furan ring joining the benzene rings, and especially the K^+ radical that by affinity to carbon 1, possible deprotonation on carbon, the final product it's also known of phenolic salt (Araújo et al., 2019b).

Recently, our group has been exploring the potential of PS-UA in the prevention and control of schistosomiasis mansoni. PS-UA showed teratogenic and toxic effects against different embryonic stages and adults of *Bimphalaria glabrata*, intermediate host of *S. mansoni* (Martins et al., 2014; Araújo et al., 2018c,d) and *in vitro* action against couples adult *S. mansoni* worms, presenting high mortality at low concentrations, as well as deep alterations in the tegument, in addition to diminishing the motility and cellular viability of the worms, while at the same time presenting lower cytotoxicity for peripheral blood monoclonal cells (Araújo et al., 2019a). In this sense, the present article aims to evaluate the *in vitro* schistosomicidal potential of PS-UA, obtained from *C. substellata*, through the parameters of motility, mortality, cell viability of the worms and tegument alterations by scanning electron microscopy, against developmental stages (5-h-old schistosomules and 21-day-old worms) of *S. mansoni*.

2. Materials and methods

2.1. Compounds

Praziquantel (purity ≥ 98%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium hydroxide was purchased from Merck KGaA (Darmstadt, Germany). All other analytical or cell culture grade reagents were purchased from Sigma-Aldrich (Brazil).

2.2. Collection of C. substellata

The species *C. substellata* (Vainio, 1887) was collected in February of 2015 in the city of Mamanguape - PB. A specimen was deposited at the Herbário Geraldo Mariz, Federal University of Pernambuco (UFPE-Brazil), with registration number 77.474, as previously described (Araújo et al., 2018c).

2.3. Extraction, isolation of usnic acid and synthesis of PS-UA

Usnic acid was isolated from *C. substellata* and the synthesis of PS-UA was performed as previously described by Araújo et al. (2018c). Briefly, a dry powder of *C. substellata* (120 g) was extracted with diethyl ether (5×) in a Soxhlet apparatus at 40 °C for 16 h. The solutions were filtered and concentrated to dryness on a rotary evaporator coupled to a water bath at 37 °C. A single fraction was isolated and purified with ethereal extract fractionated on a silica gel column (70–230 mesh), eluted in a chloroform–hexane (80:20 v/v) solvent system of increasing polarity. The fractions obtained were monitored by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) to a high purity (≥98%) compound. The molecular structure was identified by spectra of proton nuclear magnetic resonance (^{1}H NMR) and Carbon (^{13}C NMR) obtained at 400 MHz in a CDCl3 spectrometer (Varian UNITY), while infrared spectroscopy (IR) analyses were performed in a Bruker Fourier spectrometer (IFS model) with KBr disks. Subsequently, 5% KOH was added to the usnic acid until complete solubilization. The solution obtained was frozen in a −80 °C freezer, then lyophilized, and confirmation of the molecular structure of the PS-UA was given by ^{1}H NMR, IR and elemental analysis.

2.4. Parasite

S. mansoni (strain BH − Belo Horizonte, Brazil) is maintained through successive passages in *B. glabrata* and *Swiss* mice in the experimental schistosomiasis sector of the Laboratory of Immunopathology Keizo Asami (LIKA) and the Department of Tropical Medicine, both of which are part of the Federal University of Pernambuco (UFPE).

2.4.1. Preparation of S. mansoni schistosomules and PS-UA schistosomicidal assay

S. mansoni cercariae, obtained from infected *B. glabrata* after exposure to artificial light (40 °C, for 2 h), were mechanically transformed into schistosomules as previously described by Ramalho-Pinto et al. (1974). Immediately, the schistosomules were washed four times

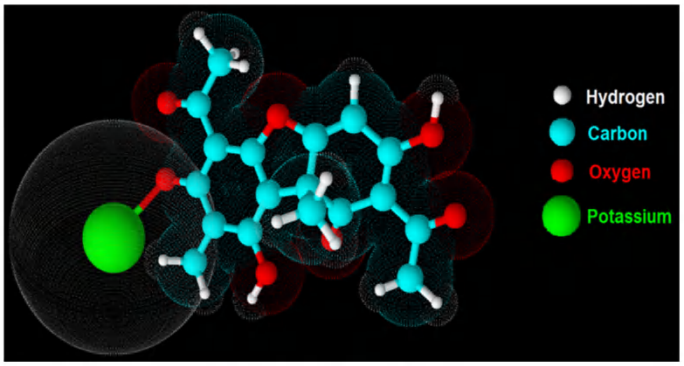


Fig. 1. Chemical structure of PS-UA.

in RPMI 1640 medium supplemented (20 mM HEPES, 100 μg/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum), distributed in 24-well culture plates with 2 mL this medium per well (50 worms/well) and incubated incubated for 5 h at 37 °C in humid atmosphere containing 5% CO_2. After this period for adaptation, schistosomules were exposed to PS-UA (solubilized in RPMI 1640 medium supplemented) to a final concentration of 100, 50, 25 and 12.5 μM. Control worms were assayed in RPMI 1640 medium supplemented as a negative control group and in 10 μM PZQ as a positive control group. Two independent experiments (200 schistosomules per concentration), were performed in quadruplicate (Pereira et al., 2015).

2.5. Ethical considerations, animals and infection

After approval of the Ethics Committee in Animal Experimentation of the Bioscience Center, UFPE (Proc. No. 23076015163/2017-65), female *Swiss* mice, weighing 28 ± 2 g, were percutaneously infected (Olivier and Stirewalt, 1952), with about 120 cercariae of *S. mansoni* (BH strain), maintained at the Schistosomiasis Experimental Laboratory of Immunopathology Keizo Asami – LIKA/UFPE. Mice were obtained and kept at the LIKA vivarium, in a controlled environment (20 ± 2 °C, 12-h daylight cycle) with free access to food (Labitum/Purina, São Paulo-SP) and water.

2.5.1. Preparation of young S. mansoni worms and schistosomicidal assay of PS-UA

Twenty-one days after infection, the mice were euthanized by cervical dislocation and the worms were aseptically recovered by perfusion of the portal system with sterile saline (0.9% NaCl w/v) (Smithers and Terry, 1965). The young worms were immediately transferred to an RPMI 1640 medium supplemented (20 mM HEPES, 100 μg/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum, being rinsed four times with this medium). Then, the worms were distributed in 24-well culture plates with 2 mL of this medium (30 worms per well), and incubated at 37 °C in a humid atmosphere containing 5% CO_2. After 2 h to enable the adaptation of the worms, PS-UA (solubilized in RPMI 1640 culture medium) was added to a final concentration of 200, 100, 50, 25 and 12.5 μM. The control worms were assayed in RPMI 1640 medium supplemented as a negative control group and in 10 μM PZQ as a positive control group. Two independent experiments (120 young worms per concentration), were performed in quadruplicate (Pereira et al., 2015).

2.6. Motility and survival of schistosomules and young worms of S. mansoni

The motility and survival of the schistosomule and young worms were evaluated with the aid of an inverted microscope (Leica DM IL Wetzlar, Germany) at 3, 6, 12 and 24 h intervals after incubation using criteria previously established by Araújo et al. (2019a). Young worms were monitored according to a decreasing viability score from 3 to 0, wherein score 3, worms that present typical movements, exhibiting peristalsis of the internal organs, suckers in movement, adhering to the bottom or sides of the culture plate, which are typical descriptions of worms of the negative control; score 2, reduced movements throughout the body, peristalsis of internal organs and suckers; score 1, movements only at the extremities or at only one of the extremities (anterior and/or posterior regions), with absence of peristalsis of internal organs and suckers not adhered to surfaces; score 0, complete absence of motions and tegument with or without changes in coloration. Meanwhile, for the schistosomule the motility scores were 3, 1.5 and 0, wherein score 3, worms that present typical movements along the body, suction cups in movement or adhered to the bottom or lateral surfaces of the plate, preserved coloration of the integument; score 1.5, absence of adhesion and reduced movement of the suction cups, movements reduced throughout the body, reduced movement only at the anterior and/or posterior extremities; score 0, absence of movements along the body with or without changes in the color of the integument. The treatment was considered lethal when no movement was observed over 2 min at both developmental stages.

2.7. Cell viability assay of young worms

The cell viability of young *S. mansoni* worms after exposure to PS-UA for 24 h was determined by the cytotoxicity assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), according to conditions previously reported by Araújo et al. (2019a). Briefly, four worms were placed in individual wells on 96-well plates containing 100 μL of MTT (5 mg/mL in phosphate-buffered saline, PBS) and incubated at 37 °C for 30 min. Thereafter, the MTT solution was replaced with 200 μL DMSO, with the purpose of dissolving the purple formazan crystals, and the optical density measured at 550 nm, in a microplate reader (M680, Bio-Rad Laboratories, Inc.). Again, as control groups, pairs of *S. mansoni* were incubated in RPMI 1640 (negative control) or exposed to 10 μM PZQ (positive control) for the same time intervals and experimental conditions. All experiments were carried out in quadruplicate and repeated at least twice. Significant differences were defined as $p < 0.05$.

2.8. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to describe the tegumentary changes due to PS-UA on the schistosomule and young worms, incubated at concentrations of 12.5 and 200 μM, respectively. At intervals of 3, 6, 12 and 24 h, both stages were sampled and fixed

with 2.5% glutaraldehyde and 4% paraformaldehyde in a 0.1 M sodium cacodylate buffer (pH 7.2) for 12 h at room temperature. Thereafter, samples were washed in the same buffer and post-fixed with 1% (w/v) OsO_4 in a 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature. Specimens were then dehydrated with increasing concentrations of ethanol (30, 50, 70, 90 and 100%) for 10 min each step. After dehydration, the critical point (HCP-2, Hitachi) was obtained for the substitution of ethanol with carbon dioxide, drying the material and mouting it on metallic stubs using double-sided carbon tape. Metallization was then performed by covering the material with a thin layer of gold for visualization and analysis with the scanning electron microscope (JEOL JSM-5600 LV).

2.9. Statistical analysis

Numerical data were analyzed with Graphpad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) and are expressed as mean ± standard deviation (SD). Statistical differences were determined by using one-way analysis of variance (ANOVA) in conjunction with Turkey's test for single-step multiple comparisons. Significant differences were defined as $p < 0.05$.

3. Results

3.1. Chemical analysis

The purified usnic acid was analyzed by HPLC and shown to have purity of 99%. Subsequently the synthesis of PS-UA was performed and its molecular structure was confirmed by ^{1}H NMR, IR and elemental analysis according to data reported by Araújo et al. (2018a; 2019a).

3.2. PS-UA and worms motility and cell viability

Tables 1 and 2 show the results of kinetics and mortality of the developmental phases of *S. mansoni* schistosomule (5 h) and young worms (21 days), respectively, exposed to PS-UA at 3, 6, 12 and 24 h intervals.

Data on schistosomules at the first observation interval, 3 h, showed concentrations of 100 and 50 μM PS-UA already caused 100% mortality, *i.e.* there was a complete absence of movements throughout the body (score 0). While, schistosomules incubated at 25 and 12.5 μM achieved 38% and 18% mortality, and 62% and 24% exhibited anterior and/or posterior movement and no adhesion of suckers to the culture plate (score 1.5), respectively. In addition, 58% of the schistosomules incubated at 12.5 μM exhibited movement at the anterior and posterior extremities and ventral sucker adhesion at the bottom or lateral sides of the plate (score 3). At 6 h, 90% of the schistosomules exposed to 25 μM had a score of 0 and 100% mortality was reached at 12 h. For the concentration of 12.5 μM the mortality of 68% and 100% was recorded at 12 h and 24 h, respectively. The schistosomules exposed to PZQ did not present any percentage of mortality at the observation intervals, all (100%) had a score of 1.5.

Meanwhile, for young worms the mortality observed at the first 3-h observation interval corresponded to 27% and 57% for the concentrations 100 and 200 μM, respectively. Meanwhile, 12 and 90% and 60 and 100% mortality rate were observed after 12 and 24 h of exposure at both concentrations, respectively. After 24 h, 40% of the worms incubated in 50 μM showed movements in only one or both extremities, with the absence of peristalsis of internal organs and suckers not adhering to the culture plate (score 1), and another 40% showed reduction of movement along the entire body and peristalsis of the internal organs (score 2) with only 20% showed typical movements, showing peristalsis of the internal organs, suction cups in movement or adhered to the bottom or sides of the culture plate (score 3), behavior similar to the worms of the negative control group. Concentrations of 25 and 12.5 μM showed no relevant motility changes. Meanwhile, all worms of the positive control group exposed to PZQ maintained a score of 1 at all other observation intervals.

PS-UA, at concentrations of 100 and 200 μM, significantly reduced formation of formazan, 50.98% and 85.87%, respectively, when compared to the negative control group. In addition, the concentrations of 100 and 200 μM of PS-UA caused greater cellular inviability of the young worms of *S. mansoni* when compared to the positive control group, worms exposed to PZQ ($^b p < 0.01$ and $^c p < 0.001$). In addition, the concentration of 200 μM of PS-UA caused greater cell mitochondrial non-viability in *S. mansoni* young worms when compared to the positive control, worms exposed to PZQ ($p < 0.001$) (Fig. 2).

3.3. PS-UA and worms ultrastructural

A kinetic analysis of ultrastructural changes was performed on schistosomules and young worms incubated at 12.5 μM and 200 μM PS-UA concentrations, respectively. Both concentrations resulted in changes in motility at all time intervals (3, 6, 12 and 24 h) and reached 100% lethality at the last observation period.

Schistosomules incubated for 24 h in supplemented RPMI 1640

Table 1
Motility score of control worms schistosomulae, treated with Praziquantel (PZQ) and with PS-UA after 3, 6, 12 and 24 h of incubation.

Groups	Mean ± standard deviation (SD) and percent of worms (%) in motility scores after incubation											
	3 h			6 h			12 h			24 h		
	0	1.5	3	0	1.5	3	0	1.5	3	0	1.5	3
Ctrl Negat.			50 ± 0.0 (100%)			50 ± 0.0 (100%)			50 ± 0.0 (100%)			50 ± 0.0 (100%)
Ctrl PZQ		50 ± 0.0 (100%)			50 ± 0.0 (100%)			50 ± 0.0 (100%)			50 ± 0.0 (100%)	
10 μM PS-UA												
100 μM	50 ± 0.0 (100%)			50 ± 0.0 (100%)			50 ± 0.0 (100%)			50 ± 0.0 (100%)		
50 μM	50 ± 0.0 (100%)			50 ± 0.0 (100%)			50 ± 0.0 (100%)			50 ± 0.0 (100%)		
25 μM	19 ± 4.24 (38%)	31 ± 5.65 (62%)		45 ± 2.82 (90%)	5.0 ± 2.82 (10%)		50 ± 0.0 (100%)			50 ± 0.0 (100%)		
12.5 μM	9 ± 1.41 (18%)	12 ± 2.82 (24%)	29 ± 4.24 (58%)	14 ± 2.82 (28%)	26 ± 4.24 (52%)	10 ± 2.82 (20%)	34 ± 5.65 (68%)	16 ± 1.41 (32%)		50 ± 0.0 (100%)		

Note: percentage values of 400 worms (200 worms schistosomules per concentration) per group. Two independent experiments.
Score 3 = Presents movement in the anterior and posterior extremities and adhesion of suction cup in the bottom or the lateral of the plate.
Score 1.5 = Presents movements in the anterior and /or posterior extremity and absence of adhesion of the suckers.
core 0 = complete absence of motions and integument with or without changes in coloration.

Table 2
Motility score of control young worms, treated with Praziquantel (PZQ) and with PS-UA after 3, 6, 12 and 24 h of incubation.

Groups	Mean ± standard deviation (SD) and percent of worms (%) in motility scores after incubation																	
	3 h				6 h				12 h				24 h					
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3		
Ctrl Negat.				30 ± 0.0 (100%)				30 ± 0.0 (100%)				30 ± 0.0 (100%)				30 ± 0.0 (100%)		
Ctrl PZQ 10 μM		30 ± 0.0 (100%)				30 ± 0.0 (100%)				30 ± 0.0 (100%)				30 ± 0.0 (100%)				
PS-UA																		
200 μM	17 ± 1.41 (57%)	10 ± 1.41 (33%)	3 ± 0.00 (10%)		23 ± 2.82 (77%)	7 ± 2.82 (23%)			27 ± 2.82 (90%)	3 ± 2.82 (10%)			30 ± 0.00 (100%)					
100 μM	8 ± 2.82 (27%)	15 ± 1.41 (50%)	7 ± 1.41 (23%)		11 ± 1.41 (37%)	15 ± 0.00 (50%)	4 ± 1.41 (13%)		14 ± 1.41 (47%)	12 ± 2.82 (40%)	4 ± 1.41 (13%)		18 ± 2.82 (60%)	12 ± 2.82 (40%)				
50 μM		4 ± 1.41 (13%)	6 ± 0.00 (20%)	20 ± 2.82 (67%)		4 ± 1.41 (13%)	6 ± 2.82 (20%)	20 ± 5.65 (67%)		7 ± 2.82 (23%)	12 ± 2.82 (40%)	11 ± 1.41 (37%)		12 ± 2.82 (40%)	12 ± 2.82 (40%)	6 ± 2.82 (20%)		
25 μM				30 ± 0.0 (100%)				30 ± 0.0 (100%)			6 ± 1.41 (20%)	24 ± 2.82 (80%)			9 ± 1.41 (30%)	00 ± 0.00 (70%)		
12.5 μM				30 ± 0.0 (100%)				30 ± 0.0 (100%)				30 ± 0.0 (100%)				30 ± 0.0 (100%)		

Note: percentage values of 120 young worms (30 pairs of worms per concentration) per group. Two independent experiments.
Score 3 = present typical movements, exhibiting peristalsis of the internal organs, suckers in movement, adhering to the bottom or sides of the culture plate.
Score 2 = present reduced movements throughout the body, peristalsis of internal organs and suckers
Score 1 = present movements only at the extremities or at only one of the extremities (anterior and/or posterior regions), with absence of peristalsis of the internal organs and not adhered suckers.
Score 0 = complete absence of motions and integument with or without changes in coloration.

Fig. 2. *In vitro* effects of PS-UA (12.5, 25, 50, 100 and 200 μM) on cell viability of *S. mansoni* young worms. Positive controls worms were treated with Praziquantel (PQZ, 10 μM). The viability was expressed as mean ± SD of the absorbance values from four experiments. $^{b}P < 0.01$ and $^{c}P < 0.001$ compared to negative control (CTRL). $^{\#c}P < 0.001$ compared to positive control (PZQ).

medium showed intact surface structure and topography (Fig. 3A–C). In Fig. 3B, we see a complete schistosomule. Fig. 3A shows the anterior region of the schistosomule with the presence of spines. The posterior portion of the schistosomule (Fig. 3C) is characterized by a ventral sucker. After 3 h at the concentration of 12.5 μM, the presence of bubbles (Fig. 4A–C) and ventral sucker invagination (Fig. 4B and C) were observed. After 6 h, numerous furrows in the region and exposure of the subtegumentar tissue in the ventral region were observed (Fig. 4D–F). At 12 h, we observed areas with cracks, furrows, loss of spicules and edema in the anterior and posterior regions (Fig. 4G–I). At 24 h, the changes show to be even more severe, reaching disintegration of the integument, deep furrows and holes (Fig. 4J–L). After 3 h of exposure to PZQ (Fig. 5A), the worms presented desquamation with the presence of holes; after 6 h blisters, muscle contraction and invagination of the ventral sucker (Fig. 5B); while after 12 h and 24 h (Fig. 5C, D) muscle contraction and shortening of the worm, areas with bumps, presence of blisters and holes were observed.

Young worms of the negative control presented preserved superficial topographies and structures, being evidenced in the anterior region ventral sucker (VS) and oral sucker (OS) (Fig. 6A–C). After 3 h, PS-UA at a concentration of 200 μM caused dorsoventral contraction (Fig. 7A–C). It is possible to visualize areas with edema, furrows, blisters, exposition of the subtegumentar tissue and desquamation after 6 h (Fig. 7D–F). While at 12 h, strong dorsoventral contraction, furrows and circular integumentary bottlenecks was observed that resulted in an extensive area with edemas (Fig. 7G–I). After 24 h, the changes were marked by holes, juxtaposed nerves, extensive integumentary erosion and corrugation of the tegument (Fig. 7J–L). The effect of PZQ on the young worms of *S. mansoni* after 3 h caused worms to be slightly curved (Fig. 8A). Effects after 6, 12 and 24 h (Fig. 8B–D) of exposure included presence of blisters, areas with swelling, muscle contraction, appearance of holes in the integument and short worms due to contraction of the longitudinal muscles.

4. Discussion

Here we report the schistosomicidal effect, *in vitro*, of PS-UA on schistosomules and young worms of *S. mansoni*. Our results show that changes in motility, mortality and ultrastructure caused by PS-UA were more pronounced than those caused by PZQ, which is currently the only drug recommended for treatment and control of schistosomiasis. PZQ, although biosecure and effective against all species of *Schistosoma* of medical interest, does not present activity against different developmental stages of the worm *S. mansoni*; hence, retreatment is necessary to kill the parasites that have since matured (Xiao et al., 1985; Caffrey,

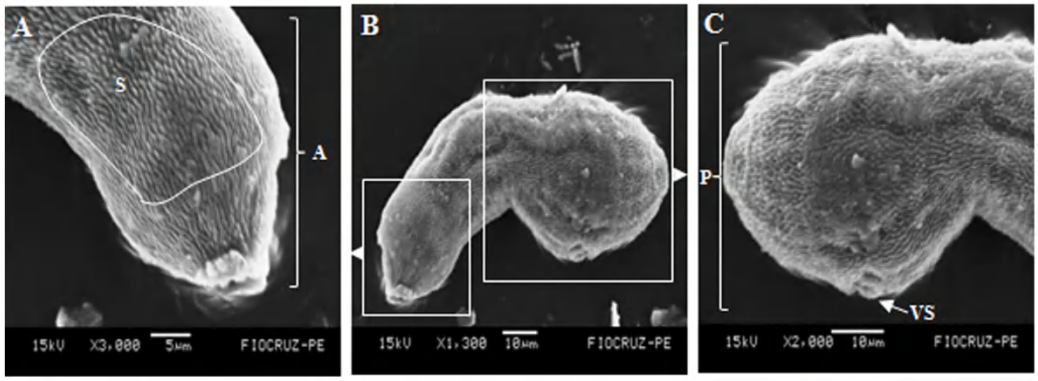


Fig. 3. (. A–C) Electromicrographs of schistosomules from the negative control group (RPMI 1640 medium supplemented). In (A, 3000×) is evidenced the anterior region (A) with the presence of spines (S). In (B, 1300×) shows the intact surface structure and normal morphological topography. In (C, 2000×) to observe the posterior region with the presence of the ventral sucker (VS).

2015). Thus, there is an urgent need to search for therapeutic alternatives that can act against different developmental stages of *S. mansoni* and thus interrupt the worm's life cycle after infection in the definitive host. Additionally, while other studies have explored the schistosomicidal activity of products of natural, semi-synthetic and synthetic origins against *S. mansoni*, the main objective of these studies is on adult worms (Aires et al., 2014b; Xavier et al., 2014, Xiao et al., 2014, El-Beshbishi et al., 2015, Mafud et al., 2018. Kapadia et al., 2017; Silva et al., 2018; Araújo et al., 2019a). Thus, there are few studies that explore the schistosomicidal activity of these products on the different developmental stages of *Schistosoma* spp.

In addition, there has been limited pharmaceutical research on chemical changes in molecules of natural origin with the objective of increasing their biological effects and reducing resistance to drugs widely used in the control of neglected diseases (Cechinel Filho and Yunes, 1998; Guido et al., 2010; Campelo et al., 2018). In this context, usnic acid has been modified into PS-UA through an acid-base reaction, where the phenolic OH is replaced by the radical K^+ (Fig. 1). This modification gives the PS-UA new physical and chemical properties, especially solubility in an aqueous medium, different from that for usnic acid which is only solubilized in organic solvents (Jin et al., 2013). Our research group explored of usnic acid and PS-UA, obtained from usnic acid, in control schistosomiasis through teratogenic and toxic effects on embryonic stages (gastrula, trochophore and veliger) and adult of *B. glabrata* (Martins et al., 2014; Araújo et al., 2018a,b,c,d). In these studies, lethal concentrations 50 and 90% for embryonic stages and adult of *B. glabrata* were lower when exposed to PS-UA compared to usnic acid. In addition, the *in vitro* schistosomicidal effect of PS-UA against couples adult *S. mansoni* (Araújo et al., 2019a) was reduced in dose and incubation time, results different from the study of Salloum et al. (2012) which explored the schistosomicidal effect of usnic acid. However, not always do chemical modifications potentiate the biological effects of some molecules. Campelo et al. (2018), who carried out chemical modifications to piplartin amide isolated from *Piper tuberculatum* to obtain five analogs and then performed *in vitro* tests against adult *S. mansoni* worms, concluded that these analogs did not present a schistosomicidal effect when compared to the study by Moraes et al (2011), where piplartin presented a promising schistosomicidal effect.

Sabah et al. (1986), while studying antimony, hycanthone, oxamniquine, niridazole, amoscanate and PZQ, all schistosomicidal agents, concluded that these drugs have low or no significant parasitic cure against different developmental stages of the worm *S. mansoni*. Similarly, when studying PZQ, Pica-Mattoccia and Cioli (2004), and Caffrey (2015) report low susceptibility of immature worms of *S. mansoni*. Oliveira et al. (2014) showed that schistosomules are not susceptible to PZQ after treatments with a dose of 40 mg/kg of PZQ, where a low reduction of only 12% in the parasitic load of the schistosomule was observed, corroborating the study by Utzinger et al. (2003). Thus, the developmental stages of *S. mansoni* that are refractory to PZQ despite shortening and Ca^{2+}-dependent contraction are evident (Fig. 5D and Fig. 8D), showing movements only at the extremities or both extremities or even paralysis similar to that observed in adult worms of *S. mansoni* (Pica-Mattoccia et al., 2008). Worms at different developmental stages recover and survive, indicating that although the initial target of toxicity is probably similar to the mechanism of action in adult worms, responses through adaptive mechanisms of the parasites allow their survival (Utzinger et al., 2003; Greenberg, 2013). These statements corroborate the data observed on the cellular viability of young worms, no significant difference was observed ($p > 0.05$) between the negative and PZQ groups (Fig. 2) and in Tables 1 and 2, as there was no mortality of schistosomules and young worms exposed to PZQ. In addition, discrete tegumentary changes were observed in the schistosomules and young worms exposed to PZQ (Figs. 5 and 8), differently from the alterations resulting from the exposure of both developmental phases to PS-UA (Figs. 4 and 7).

A study by Pereira et al. (2015) evaluated (−)-6,6′-dinitrohinokinin (DNK) on the developmental stages of *S. mansoni* and observed that DNK was more toxic for schistosomules and adult worms compared to young worms at the 24 h interval, with LC_{50} values of 57.4, 103.9 and 522.8 µM, respectively. These results corroborate what has been reported for adult *S. mansoni* worms exposed to PS-UA (Araújo et al., 2019a) and reported here, where *S. mansoni* worms showed higher susceptibility to PS-UA at the stages of schistosomules and adult worm. According to Hines-Kay et al. (2012), the lower susceptibility of young *S. mansoni* worms to PZQ is related to transcriptional supra-regulation of their genes when compared to adult worms. These genes are responsible for the escape mechanism that encode the multiple drug transporter, as well as calcium regulators, stress and related cellular apoptosis of the parasite; this results in greater susceptibility of young worms to PZQ. Mei and LoVerde (1997) compared levels of enzymes that neutralize reactive oxygen species (cytosolic Cu–Zn, superoxide dismutase (CT-SOD), signal-peptide-containing SOD (SP-SOD), glutathione peroxidase (GPX), and glutathione transferase (GST)), in schistosomules and adult worms of *S. mansoni* and observed that adult worms had higher enzyme levels, reaching levels approximately 100 times higher than those found in schistosomules. These findings may explain why PS-UA has been shown to be more effective at 12.5 µM in

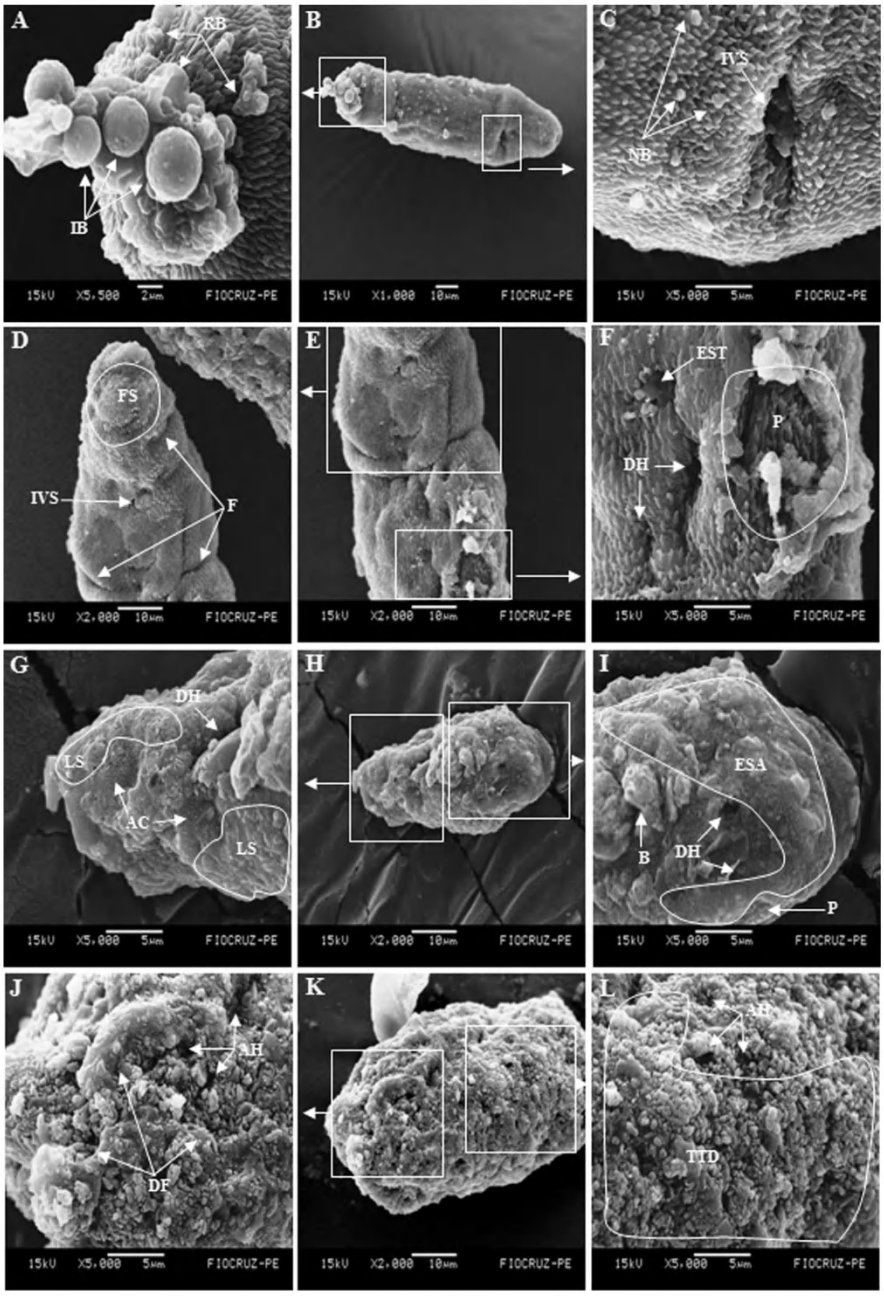


Fig. 4. (. A–L) Electromicrographs of schistosomules exposed for 3 h to 12.5 μM PS-UA. After 3 h of exposure (A–C). In B (1000×) ventral view with morphological alteration. Enlarged anterior region (A, 5500×), with bubbles of varying sizes, some intact bubbles (IB) and ruptured bubbles (RB). In C (5000×) observe invagination of the ventral sucker (VS). In the interval of 6 h (D–F). In E (2000×), we observed changes in the medial and posterior regions of the schistosomule, in vision enlarged view of the posterior region (D, 2000×), showing the invaginated ventral sucker (IVS), presence of furrows (F) and extremity a focal swelling (FS). F (5000×) in the middle region shows peeling areas (P), exposure of the subsegmental tissue (EST) and deep holes presence (DH). After 12 h of exposure (G–I). In H (2000) shortening of the schistosomule was observed, with visible changes in the enlarged picture (G, 5000×) as areas with cracks (AC), loss spicules (LS), deep holes (DH). In I (5000×) observe presence of bubbles (B), extensive swelling area (ESA), peeling (P) and deep holes (DH). At 24 h exposure interval (J–L). (5000×) and L (5000×) evidenced the presence of holes (AH), with deep furrows (DF), and total tegument disintegration (TTD) with visible holes (AH) respectively.

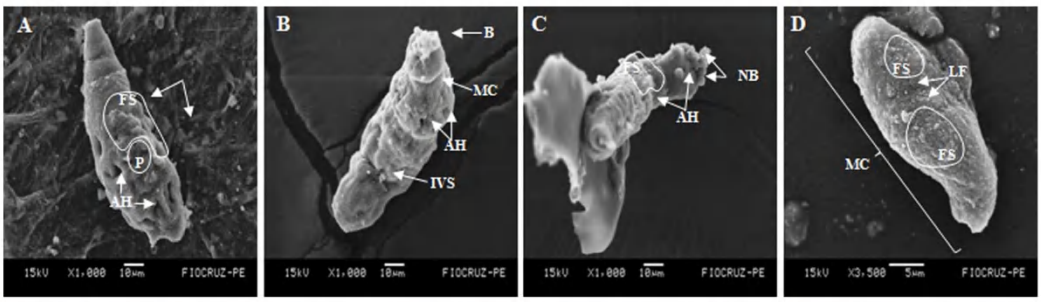


Fig. 5. (. A–D) Electromicrographs of schistosomules exposed to PZQ (10 µM). After 3 h of exposure (A, 1000×) it was observed peeling (P), presence of holes (AH) and areas with swellings (FS) still in the median region of the schistosomule. After 6 h B (1000×) muscle contraction (MC) is observed in the schistosomule with the appearance of bubbles (B) in the anterior region, and the presence of holes (AH) and the invagination of the ventral sucker (VS) are also evident. In the interval of 12 h C (1000×), there are numerous bubbles (NB) in the anterior region of the schistosomule, while in the medial region there are areas with swelling (FS) and the presence of holes (AH). After 24 h (D, 3500×) strong muscular contraction is observed with shortening of the schistosomule (MC), with focal swelling (FS) and low furrows (LF).

the mortality of schistosomules, as reported here, than in the adult *S. mansoni* worm populations exposed to PS-UA for 24 h under the same treatment protocol as reported by Araújo et al. (2019a).

The *S. mansoni* integument is an important target for the development of new schistosomicidal drugs because it plays a vital role in the parasite (nutrient absorption, antioxidant systems, immune evasion, excretion, regulation of osmotic pressure and signal transduction) the integument is an important target in the study of drugs with potential schistosomicidal activity (Mei and Lo Verde, 1997; Hines-Kay et al., 2012; Skelly et al., 2014; Sotillo et al., 2015). According to Fig. 4(B, E, H and K) and Fig. 7(C, F, I, J and L), PS-UA caused extensive and severe changes in patterns at both developmental stages, in a dose- and time-dependent manner. As suggested by Araújo et al. (2019a) and based on the results presented here, we believe that when exploring the effect of PS-UA in an experimental model *in vivo,* against different developmental stages of *S. mansoni*, tegument changes may favor the exposure of antigens on the surface of the worms, signaling the immune response of the intermediate host and thereby complementing the schistosomicidal action of PS-UA. This hypothesis is corroborated in the studies conducted by Eissa et al. (2011, 2015) and El-moslemany et al. (2016) when exploring the schistosomicidal potential of Miltefosine against different developmental stages of *S. mansoni* in experimental model *in vitro* and *in vivo*.

Although the mechanism of action of PS-UA has not yet been elucidated, high susceptibility of the developmental stages of *S. mansoni* has been demonstrated in this work. Hockley (1973), Roberts and Costelo (2003), Joseph et al. (2009) and Araújo et al. (2019a) have corroborated the *in vitro* effects of PS-UA on couples *S. mansoni* in terms of muscle function (motor activity) and tegumental destruction, with observed changes being more intense and extensive in male worms than in females. This is possibly associated with the greater amount of mitochondria present in the tegument of male worms, causing redox effects associated with membrane permeability and mainly altering the energy metabolism of the parasites with the decoupling of electron transport chains. However, in spite of all the biotechnological advances (molecular biology, genomics, medical and analytical chemistry, mathematical and computational modeling to measure the mechanisms of action of schistosomicidal drugs), the discovery of new drugs for the treatment of schistosomiasis that affect all the developmental phases of *Schistosoma* spp. continues to be a major challenge, because, in addition to the schistosomicidal action, new drug candidates need to present tolerable limits of toxicity (Campelo et al., 2018; Mafud et al., 2018).

In this context, the cytotoxic and toxicological aspects of PS-UA were studied. PS-UA exhibited cytotoxicity to peripheral blood monoclonal cells (PBMC) with selectivity indices, exhibiting IC_{50} >200 µM. Meanwhile, in terms of acute toxicity in a murine experimental model, according to the requirements of the international parameters, PS-UA at the concentration of 500 mg/kg did not present hematological, biochemical and histopathological alterations. However, at the concentration of 2000 mg/kg, the kidney and liver were the main organs compromised, the spleen remained preserved, yet the LC_{50} was not determined as only 40% mortality was reached at this concentration (Araújo et al., 2019b). Thus, experiments to evaluate PS-UA in a murine experimental model infected with *S. mansoni* would also be important, which would investigate the schistosomicidal effect *in vivo* and analyze homeostatic, parasitological and histopathological parameters in detail.

5. Conclusion

The results of this study indicated greater efficacy of PS-UA on the developmental stages of *S. mansoni* than praziquantel (PZQ), a drug adopted by the World Health Organization. In addition, the results

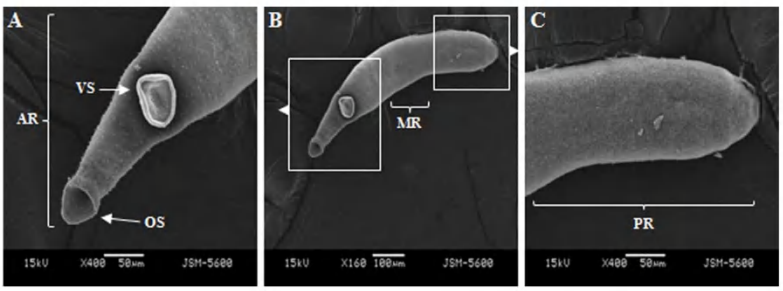
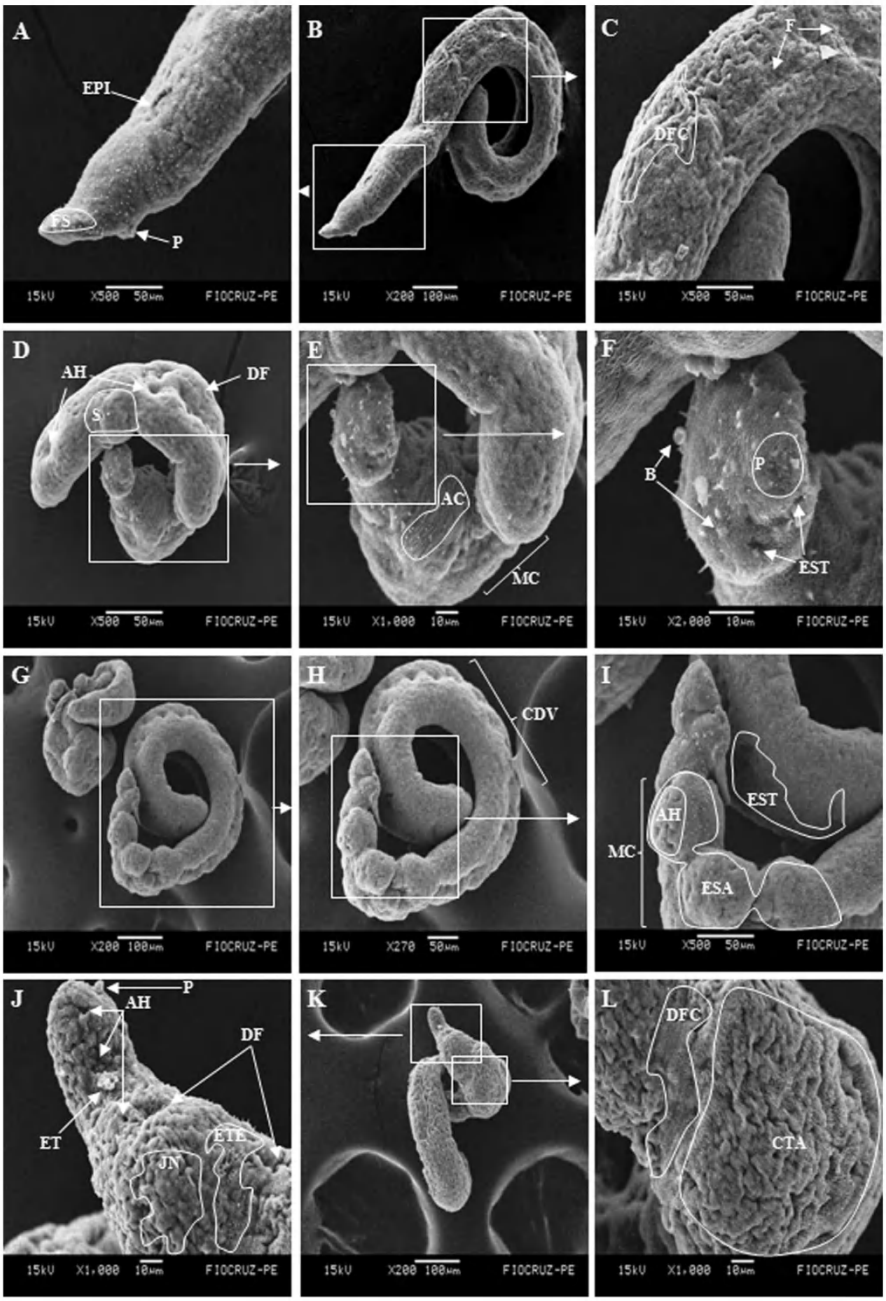


Fig. 6. (. A–C) Electromicrographs of young worms from the negative control group (RPMI 1640 medium supplemented). In B (160×) intact surface structure and normal morphological topography of young worm, medium region (MR). (A, 400×) enlarged view of the anterior region (AR) showing oral sucker (OS) and ventral sucker (VS). In C (400×) the posterior region (PR) of the young worm is observed without alterations.



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obtained through SEM demonstrate the ultrastructural changes in which PS-UA caused more tegumentary alterations than PZQ. Therefore, considering the urgent need for new drugs and the schistosomicidal effect presented by PS-UA, this molecule may represent a new compound for the future treatment of schistosomiasis mansoni.

CRediT authorship contribution statement

Hallysson D.A. Araújo: Writing - review & editing. **Victor H.B. Santos:** . **Fábio A. Brayner:** . **Luiz C. Alves:** . **Nicácio H. Silva:** . **Mônica C.P.A. Albuquerque:** . **André L. Aires:** . **Vera L.M. Lima:** .

Fig. 7. (. A–L) Electromicrographs of young worms exposed for 3 h at 200 μM PS-UA. After 3 h of exposure (A–C). In B (200×) young worm with slight dorsoventral curvature. In A (500×) in the anterior dorsal region there is extensive peeling of the integument (EPI), with slight peeling (P) of the ventral region. In C (500×) there is in the median region of the worm young furrows (F) and deep folds coalescing (DFC). In the interval of 6 h (D–F). In D (500×) the young worm evidences a more accentuated dorsoventral curvature with presence of holes (AH), deep furrows (DF) and areas with swelling (S), in enlarged vision (E 1000×), and (F 2000) is (B), peeling (P), and exposure of the subtegumental tissue (EST), respectively. In the present study, it was possible to observe in the anterior region areas with cracks (AC), mild muscle contraction (MC) and presence of bubbles (B). After 12 h (G-1). In G (200×) and H (270×) the young worm is found with dorsoventral curvature, in an enlarged view (I, 500×), it is possible to observe extensive swollen areas (ESA) with presence of holes (AH), exposure subtegumental tissue and muscular contractions (MC). The interval of 24 h (J–L). In K (200×) shows young worm in spiral form, in an enlarged view of the anterior region (J, 1000×) there is extensive tegmental erosion (ETE), eruption of the tegument (ET), peeling (P), presence of holes (AH), deep furrows (DF) and juxtaposed nerves (JN). In L (1000×) in the median dorsal region evidence deep folds coalescing (DFC) and corrugation of the tegument area (CTA).

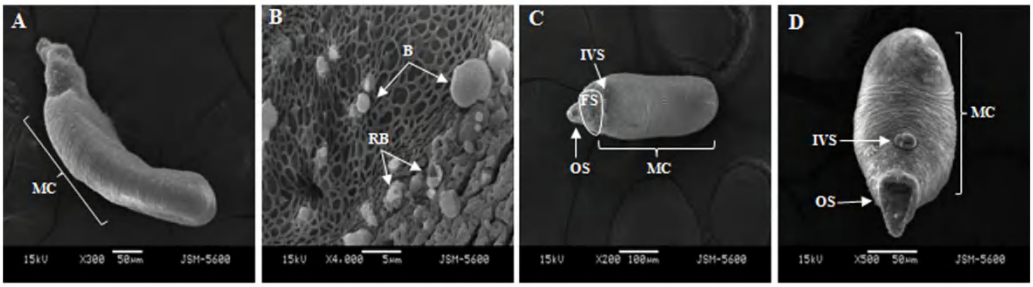

Fig. 8. (. A–D) Electromicrographs of young worms exposed to PZQ (10μM). After 3 h of exposure (A, 300×) a slight muscular contraction (MC) of the worm is observed. In the interval of 6 h in B (4000×) in the anterior region observe the presence of bubbles (B) with some ruptured bubbles (RB). After 12 h C (200×) and 24 h D (500×) in the anterior region of the worm, we observed the oral sucker (OS), followed by an area with focal swelling (FS) and invagination of ventral sucker (IVS), as well as muscle contraction (MC) present in both intervals, being even more evident this last change in the last observation interval.

Declaration of Competing Interest

The authors have no conflicts of interest.

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3.6 ARTIGO 6

USNIC ACID POTASSIUM SALT: EVALUATION OF THE ACUTE TOXICITY AND ANTINOCICEPTIVE EFFECT IN MURINE MODEL

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Usnic Acid Potassium Salt: Evaluation of the Acute Toxicity and Antinociceptive Effect in Murine Model

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Abstract: To obtain usnic acid potassium salt (PS-UA), the usnic acid (UA) was extracted and purified from the lichen *Cladonia substellata*, and modified to produce PS-UA. The structure was determined by ^{1}H-NMR, IR and elemental analysis, ratified through computational models, as well as identification the site of K^{+} insertion in the molecule. Antinociceptive activity was detected through contortions in mice induced by acetic acid and formalin (phases I and II) after treatments with 10 and 20 mg/kg of PS-UA, indicating interference in both non-inflammatory and inflammatory pain. After oral administration at doses of 500, 1000 and 2000 mg/kg, no deaths of mice with treatments below 2000 mg/kg were observed. Except for body weight gain, food and water consumption decreased with treatments of 1000 and 2000 mg/kg, and the number of segmented leukocytes was higher for both treatments. Regarding serum levels, cholesterol and triglycerides decreased, however, there was an increase in hepatic transaminases with both treatments. Liver and kidney histological changes were detected in treatments of 2000 mg/kg, while the spleen was preserved. The PS-UA demonstrated antinociceptive activity while the acute toxicity

at the concentration of 2000 mg/kg was the only dose that presented morphological changes in the liver and kidney.

Keywords: lichen; *Cladonia substellata*; usnic acid derivatives; antinociceptive activity (phase I and II); toxicological survey; soluble drug; histopathology

1. Introduction

The lichen biota constitutes about 8% of total vegetation and is used in different cultures around the world [1]. Lichens present in their symbiotic structure at least one fungus (heterotrophic mycobiont) and one or more algae or cyanobacteria (autotrophic photobiont), which produce secondary metabolites. About 1000 lichen metabolites have been described, of which more than 80% are exclusive to the mononuclear, aromatic, depsidone, diphenyl ether, and dibenzofuran classes [2].

Ethnopharmacological research has listed the use of lichens and their derivatives in folk medicine for a wide range of pharmacological activities, demonstrating astringent, laxative, anticonvulsant, antiemetic, antiasthmatic, anti-inflammatory, and antibiotic properties, as well as uses against diarrheal diseases, skin diseases, epilepsy, convulsions, sore throats, and toothaches and also for the treatment of cardiovascular, respiratory, and gastric diseases [2,3]. The great interest in finding remedies from natural resources is directly associated with the high cost of synthetic drugs, which mainly affect economically disadvantaged populations seeking primary care in poor or developing countries [4].

Among the secondary metabolites of lichens, there is usnic acid (UA) (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione $C_{18}H_{16}O_7$), which has a yellow color and is metabolized by several species widely distributed in tropical and subtropical countries [5]. UA is naturally found in two enantiomeric forms, differentiated by the orientation of the methyl group located at position 9b. UA is also attributed with a variety of pharmacological potential, having gastroprotective, immunostimulatory, antiviral, antimicrobial, anti-inflammatory, antiprotozoal and antitumor activities [1,6,7].

In addition, pharmacological studies have been carried out to test and develop natural compounds isolated from lichens for human use related to pain, which is a neurological sign with nociceptive perception due to unpleasant sensations stimulated by subjective and objective factors of the system. Both factors are directly related to the mechanisms of pain response in stimulation to peripheral nerve responses (pain in the first minutes) or more pronounced stimulation (pain lasting ≥ 10 min after injury) [8]. In this regard, Okuyama et al. [9] evaluated diffractaic acid and UA in a murine pain model and observed that UA had a very considerable effect when using the acetic acid contortion method. However, UA has the disadvantage of presenting low aqueous solubility and high toxicity due to its hydrophobic characteristics related to its physico-chemical properties [10]. As a result, several studies have aimed at improving the properties of UA, such as the inclusion of usnic acid β-cyclodextrin [11], usnic acid-polyacrylamide [12], solid lipid nanoparticles [13], PLGA microsphere encapsulation [14], and the incorporation in polyurethanes [15,16] and in liposomes [17].

Therefore, it is important to introduce new, effective methods to increase the solubility and dissolution rate of drug candidates in order to improve their oral bioavailability, to increase the predictability of responses, and/or to reduce the dose [18–20]. Thus, one of the alternatives for increasing the bioavailability of UA is to chemically modify it in the form of usnic acid potassium salt (PS-UA), which optimizes not only its solubility but also its toxicity without decreasing its biological potential [21–25]. Although lichen substances are widely mentioned in folk medicine, studies of their chemically modified bioactive molecules are incipient. UA in the form of PS-UA, inhibits invasion and metastasis in colorectal cancer [21]; its efficiency against adult snails [22] and different embryonic stages of *Biomphalaria glabrata* (vector of schistosomiasis) [23,24] has been demonstrated, as well as good in vitro activity against adult couples worms of *Schistosoma mansoni* at concentrations of 100

and 50 μM in a 24-h interval. With respect to cytotoxicity, PS-UA was non-toxic to peripheral blood mononuclear cells (PBMC) at the same concentrations [25].

However, effects on pain, as well as its acute toxicity, have not yet been reported. In this sense, the objective of this study was to evaluate, for the first time, the antinociceptive activity of PS-UA and its action mechanism through the nociceptive pathway and to determine its acute toxicity in a murine model by analyzing the behavioral, hematological, biochemical and histological parameters.

2. Results and Discussion

2.1. Chemical Analysis of UA and PS-UA

UA TLC analysis showed a Rf value of 0.84, while in the HPLC the retention time (RT) was 20.46 minutes, consistent with the standard UA RT. The chemical structures of UA and PS-UA were confirmed by ^{1}H-NMR, ^{13}C-NMR (400 MHz, Acetone-d6) and IR (KBr) and ^{1}H-NMR, IR and elemental analysis respectively (see supplementary data). The optical rotation of UA was α_{25}^{D} +478.2200 (c 1.0 acetone). In this way, the UA used in our study is dextrorotary.

PS-UA is characterized by the presence of K^{+} counterion, what leaded to be UA a water-soluble derivative. Huneck and Yoshimura [26] described the PS-UA as a phenolic salt, having its first deprotonation at the OH group in C1 (Figure 1). Guo et al. [1] had mapped the pKa of OH groups of UA molecule, and they also considered the most acid group should be the OH in C1. However, crystallographic evidences mentioned by Ribár et al. [27] suggest the possibility of linkage of the potassium counterion to the beta-diketone group. In both cases, the ressonance of keto groups could desplace the charge, leading the proposed structure for the salt.

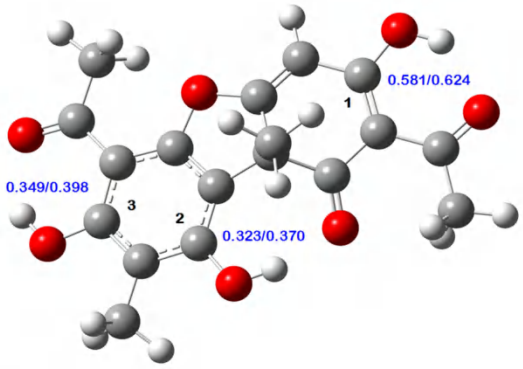

Figure 1. Structure of the usnic acid (most stable tautomer) from AM1 geometry optimization. The carbons bonded to hydroxyl groups, and the respective ESP (electrostatic potential) charges (B3LYP/6-31g+(d,p)) of each (ideal gas/water) are indicated.

In order to confirm the PS-UA structure, we performed some calculations using the Gaussian 09 program [28]. The geometry optimization of tautomer UA, which is the most stable according to previous theoretical studies [29,30], was performed using the AM1 semi-empirical method, and the electronic part of equilibrium geometries was obtained in accordance with the ab initio level of theory. The same procedure was applied to the anions of UA, formed after each possible deprotonation (Figure 1), with or without a previous proton abstraction, in both ideal gas and water.

The optimized geometry of UA tautomer of usnic acid is presented in Figure 1, which is similar to previous calculations [30]. From the ESP (electrostatic potential) charges also presented in this figure, we observe that carbon 1 is the most electrophylic as compared to carbons 2 or 3, in both ideal gas and aqueous solution. This indicates a first deprotonation in the hydroxyl group bonded to this carbon, since it can more effectively stabilize the oxygen charge after anion formation. In order to

confirm this preference in terms of the stability of each anion, we compared the sum of the electronic energy (from B3LYP) with the thermal correction term (from AM1) of each anion of UA, formed by deprotonation on carbons 1, 2 or 3 (leading to the anions A1, A2, and A3, respectively) according to the numbering on Figure 1. Both in ideal gas and in aqueous solution, the anion created by the deprotonation of the nonphenolic OH is the most stable. The following orders of stability, with the energies relative to such anion, were found: (a) A1 > A2 (6.76 kcal mol^{-1}) > A3 (18.23 kcal mol^{-1}) in ideal gas and (b) A1 > A2 (7.81 kcal mol^{-1}) > A3 (10.94 kcal mol^{-1}) in water. These data support the hypothesis that the most acid proton belongs to the OH group bounded with carbon 1, as previous calculations using the PCM implicit solvent model have verified [30].

After the first deprotonation, the second can occur on carbon 2 (leading to anion A12) or carbon 3 (leading to anion A13). It was found that the anion A12 is more stable than A13, by a difference of 2.28 kcal mol^{-1} in ideal gas and 1.72 kcal mol^{-1} in water. Therefore, the anion formation order (as a result of sequential deprotonation) should be A1 → A12 → A13, where A13 is the anion which charge -3, the result of three successive deprotonations. The same trend was verified by Galasso [30]. This implies the probable position for K^+ in the usnic acid salt near the negatively charged oxygen bonded to carbon 1 (Figure 2).

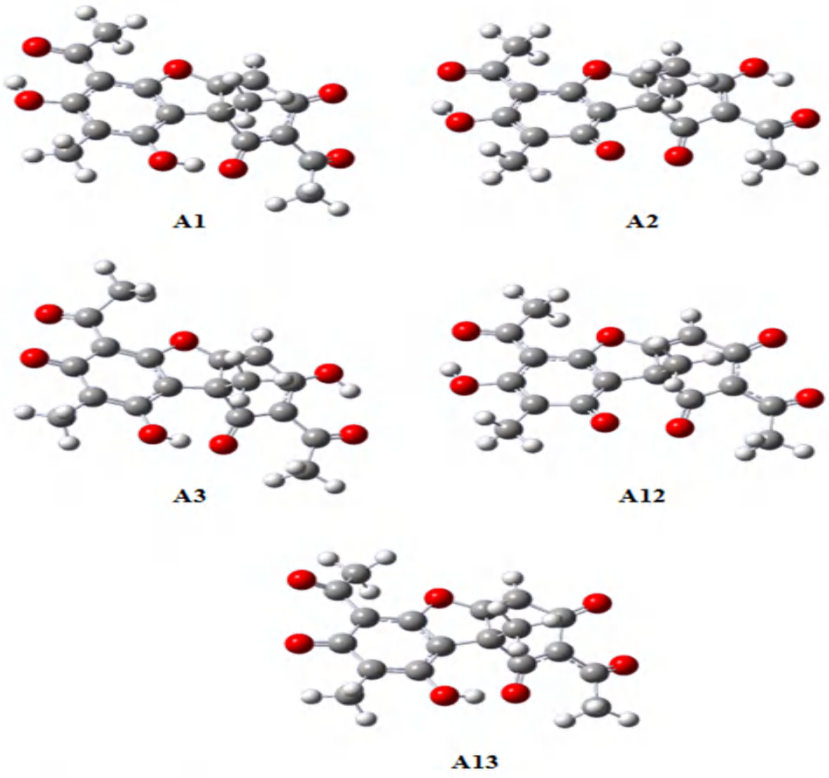

Figure 2. Equilibrium structures of usnic acid anions. A1, A2 and A3 formed after one single deprotonation and anions A12 and A13 formed after two deprotonations.

Natural derivatives are important sources of raw materials in obtaining molecules in the composition and development of promissory compounds, when the aim is the development of new drugs. In addition, they can also allow structural modifications (syntheses) from these substances, potentiating their biological activity, thus leading to an optimization of therapeutic activity [4,18,21–25,31,32]. Therefore, we contribute with the first report of PS-UA obtained from UA isolated from *Cladonia substellata* (lichen). This derivative showed low acute toxicity and high antinociceptive activity at low concentrations in a murine model. This result, in addition to being a novelty, can be considered as useful for further studies that involve tropical and neglected diseases.

2.2. *Signs of Toxicity and Behavioral Analysis of Mice after PS-UA Treatment*

The toxic effects of PS-UA through behavioral analyses in mice are shown in Table 1.

Table 1. The parameters related to toxic signs and the behavioral analysis in swiss female mice assessed after the oral administration of the PS-UA in doses of 500, 1000, and 2000 mg/kg.

Parameters	Treatments PS-UA (mg/kg)			
	Control	500	1000	2000
Stimulants				
Increased respiratory frequency	-	+	+	+++
Piloerection	-	++	++	++
Stereotyped movement	-	-	+	++
Fine tremors	-	-	-	+
Lifting upper train	-	+	+	+
Depressant				
Prostration	-	+++	+++	+++
Lowering hind quarters	-	-	-	++
Others				
Photophobia	-	-	-	+
Spasms	-	-	-	+
Fecal excretion	-	-	+	+
Abdominal distension	-	-	-	++
Change: Depression x Shake				
Death	-	-	-	++

- = no effect; + = low effect; ++ = moderate effect; and +++ = high effect.

The acute toxicity assay is usually performed to safely determine the dose ranges of substances, but it can also provide initial information on the mechanisms of toxicity or homeostatic changes [33]. The negative control group did not show behavioral changes, whereas all tested concentrations (500, 1000 and 2000 mg/kg of PS-UA) showed at least some effect, either stimulant, depressant or change in depression versus agitation. According to Nascimento et al. [34], systemic toxicity is demonstrated through alterations, emphasizing those that affect both the central and the autonomous nervous system, as well as somatomotor activity. Moderate and high piloerection and prostration effects were observed in the initial period after administration of PS-UA at 500, 1000, and 2000 mg/kg. On the other hand, the reversion of these initial clinic signals after 1 h of administration was observed.

Mice treated with 2000 mg/kg showed the highest number of behavioral changes and were the only ones with mortality at 48 and 72 h intervals, but this was only a 40% mortality rate. In this context, we can observe that the PS-UA LD_{50} (although it was not possible to calculate) was shown to be higher than that of UA 838 mg/kg as reported by Sigma-Aldrich [35]. These results corroborate with the Organization for Economic Cooperation and Development (OECD) technical specifications [36], which indicate that substances with an LD_{50} of more than 1000 mg/kg orally are considered safe or slightly toxic. Thus, we can observe that after the transformation of UA into PS-UA, the result was a drug with greater bioavailability and a much lower toxic effect.

The toxicity mechanism of UA has not yet been completely elucidated. However, Han et al. [37], Pramyothin et al. [38], and Joseph et al. [39] have already signaled that UA acts by altering the integrity of the cellular membrane (lipophlilic characteristic of the drug), allowing the liberation of hepatospecific enzymes, mainly transaminases. In addition, it causes the destruction of mitochondrial function (complex I to IV of electron transporting chain), exhibiting the loss of control of the cell respiration and synthesis of ATP. These studies indicated that UA has a similar effect to that of carbon tetrachloride, which evolves the generation of free radicals, which results in injuries in both cell and mitochondrial membranes, expression of genes associated to lipid peroxidation, Krebs cycle, and apoptosis, increasing the production of reactive oxygen through the electron transporting chain, leading to cell death.

In the USA, these aforementioned hepatocellular damages were confirmed after using LipoKinetix®, a dietary supplement containing UA. The users who consumed this supplement showed liver acute collapse, one needing a hepatic transplant. Another patient, after 8 weeks, had recovered due to specialized accompaniment [40]. Similarly, two other users that had consumed three capsules a day of UDP-1 (dietary supplement with 150 mg of UA, 525 mg of carnitine, and 1050 mg of calcium pyruvate per capsule) developed severe hepatoxicity after three months of use and exhibited fulminant hepatic failure. In one of the cases, liver transplant was necessary. Histopathological analysis showed cytoplasmic lymphocytic infiltrates and areas with necrosis in the liver of patients who had used UDP-1 [41].

No significant differences ($p > 0.05$) in body weight gain were observed for any treatment. In relation to food and water consumption, it was observed that only the mice in the control group and treated with 500 mg/kg did not present a significant difference. In contrast, mice treated with PS-UA at 1000 and 2000 mg/kg showed a reduction ($p < 0.001$) in food consumption and less water consumption than control mice Table 2. This may be directly related to the metabolic changes observed during treatment.

Table 2. The water and food consumption of mice from the controls and those treated with PS-UA.

Parameters	Treatments PS-UA (mg/kg)			
	Control	500	1000	2000
Water consumed (mL)	38.75 ± 1.49	34.10 ± 2.29	27.30 ± 7.09 [a]	19.40 ± 6.11 [a]
Food consumed (g)	33.70 ± 1.29	30.20 ± 3.56	22.85 ± 3.40 [a]	15.95 ± 7.59 [a]
Weight gain (g)	32.40 ± 1.57	31.66 ± 1.50	31.60 ± 2.15	31.88 ± 1.84

Significantly different from the control: [a] ($p < 0.001$) for the 1000 and 2000 mg/kg. Data are the means ± standard deviations.

The evaluation of these parameters is of great importance, mainly for substances with therapeutic purposes such as analgesics and anti-inflammatory agents, since adequate intake of nutrients and water are essential for the potentiality of the drug and/or to avoid gastrointestinal irritations mainly caused by the administration of these drugs over a prolonged period [42]. At present, we already have in-depth studies with humans on these mechanisms where the objective was to more precisely demonstrate drug nutrient interactions and their effects on the bioavailability of the drugs [43,44].

2.3. Haematological and Biochemical Analyses

The hematopoietic system is one of the most sensitive targets for toxic compounds and serves as an important indicator, being reliable to evaluate health and safety conditions and very well defined for the pathophysiological changes of blood hemostasis in both humans and the murine model [33]. In this report, both the red blood cell and leukocyte profiles were analyzed as important toxicological indices. The results described in Table 3 show that PS-UA did not cause severe alterations (hyperchromic/hypochromic, microcytic/macrocytic and anemia) in the red series.

Table 3. The hematological parameters of the blood of mice treated with PS-UA.

Parameters	Treatments PS-UA (mg/Kg)			
	Control	500	1000	2000
Erythrocytes (10^6/mm^3)	8.81 ± 0.81	8.88 ± 0.26	8.90 ± 0.41	9.16 ± 0.72
Hematocrit (%)	45.75 ± 3.97	45.55 ± 1.12	45.65 ± 2.65	47.05 ± 3.74
Hemoglobin (g/dL)	15.81 ± 1.40	15.53 ± 0.36	15.37 ± 0.71	15.76 ± 1.01
MCV (fL)	51.90 ± 2.23	51.25 ± 1.46	52.10 ± 0.82	51.28 ± 1.89
MCH (pg)	17.90 ± 0.38	17.35 ± 0.54	17.23 ± 0.17	17.15 ± 0.41
MCHC (%)	34.25 ± 0.95	33.25 ± 0.50	33.25 ± 0.50	33.00 ± 0.81
Leukocytes (10^3/mm^3)	2.21 ± 0.49	2.99 ± 0.52	3.65 ± 0.63 [a]	4.52 ± 0.96 [b]
Segmented (%)	40.75 ± 9.35	66.50 ± 21.75	75.75 ± 12.61[a]	74.25 ± 8.53 [a]
Lymphocytes (%)	21.03 ± 8.41	26.70 ± 4.67	28.65 ± 8.93	20.25 ± 2.14
Monocytes (%)	14.33 ± 4.93	12.33 ± 4.04	16.67 ± 2.88	14.83 ± 2.46

Significantly different from the control: [a] ($p < 0.05$) and [b] ($p < 0.001$) from the 1000 and 2000 mg/kg treatments. Data are the means ± standard deviations. MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; and MCHC: mean corpuscular hemoglobin concentration.

However, it stimulated the immune system, with an increase in segmented cells for treatments of 1000 and 2000 mg/kg. This increase in the number of segmented leukocytes has also been reported in other studies evaluating the acute toxicity of organic extracts [45,46]. Recently, a study by Oliveira et al. [47], evaluating the acute toxicity of *Pilosocereus gounellei* saline extract on mice, observed a significant increase in the number of segmented cells at a 2000 mg/kg treatment and total leukocytes at 5,000 mg/kg. According to Kumar et al. [48], the leukogram is the part of the hemogram that investigates quantitative and/or morphological alterations of the leukocyte series. Its quantitative abnormalities are leukocytosis (increase) and leukopenia (decrease). Leukocytosis can be attributed to physiological and/or pathological factors such as tissue damage (inflammation) in the example of the recurrent liver of the exacerbated metabolism of substances which at very high concentrations present toxicity.

The results of the biochemical analyses are described in Table 4. The treatments with PS-UA revealed decreased serum levels of cholesterol and triglycerides in the 1000 and 2000 mg/kg treatments, compared to the control group. This decrease can be directly related to the decrease of food intake and is not necessarily caused by metabolic changes (Table 2). Meanwhile, the values of the biochemical levels of ALT (alanine aminotransferase), AST (aspartate aminotransferase), alkaline phosphatase, and creatinine increased and varied significantly ($p < 0.05$) between the groups for the 1000 and 2000 mg/kg treatments (Table 4). These increased serum levels indicate liver hepatotoxicity and impaired renal function for PS-UA.

Table 4. The biochemical parameters of the blood of mice treated with PS-UA.

Parameter	Treatments PS-UA (mg/kg)			
	Control	500	1000	2000
Albumin (g/dL)	2.02 ± 0.28	1.90 ± 0.19	1.75 ± 0.09	1.80 ± 0.18
ALT (U/L)	39.46 ± 2.94	63.39 ± 20.7	86.78 ± 7.74 [b]	207.6 ± 25.00 [c]
AST (U/L)	115.8 ± 6.03	143.3 ± 22.5	229.44 ± 45.1 [a]	306.4 ± 19.4 [c]
Total protein (g/dL)	5.22 ± 0.05	5.27 ± 0.13	4.85 ± 0.24 [b]	4.87 ± 0.12 [b]
Alkaline phosphatase (IU/L)	15.04 ± 2.65	19.80 ± 4.55	31.00 ± 9.39 [b]	33.65 ± 6.74 [c]
Urea (mg/dL)	45.62 ± 2.54	46.56 ± 2.17	47.65 ± 1.27	46.43 ± .2.25
Creatinine (mg/dL)	041.20 ± 0.06	041.60 ± 0.06	053.40 ± 0.02	064.60 ± 0.12 [c]
Total cholesterol (mg/dL)	90.40 ± 11.95	92.60 ± 15.99	62.20 ± 14.45 [b]	60.50 ± 3.10 [b]
Triglycerides (mg/dL)	133.4 ± 27.36	112.8 ± 11.82	54.50 ± 7.55 [c]	51.75 ± 8.13 [c]

Significantly different from the control: [a] ($p < 0.05$), [b] ($p < 0.01$), and [c] ($p < 0.001$) from the 1000 and 2000 mg/kg treatments. Data are the means ± standard deviations. ALT: alanine aminotransferase and AST: aspartate aminotransferase.

2.4. Histopathological Analyses of the PS-UA Treatments

The PS-UA treatments did not show statistically significant differences ($p > 0.5$) for liver, kidney, and spleen weights at doses of 500 and 1000 mg/kg compared to the control group: The control group was 1.51 ± 0.14, 0.31 ± 0.03, and 0.12 ± 0.25; PS-UA 500 mg/kg was 1.53 ± 0.19, 0.31 ± 0.01, and 0.11 ± 0.01; and PS-UA 1000 mg/kg was 1.83 ± 0.22, 0.33 ± 0.46, and 0.11 ± 0.03. However, in the treatment with 2000 mg/kg PS-UA, there were changes in the liver and kidney morphologies (increase in size/weight) compared to the control group (3.06 ± 0.42 ($p < 0.001$) and 0.44 ± 0.03 ($p < 0.001$)). Only the spleen weight (0.12 ± 0.02) remained similar to the control.

The histopathological analysis of liver, kidney, and spleen for the control group and the 500, 1000, and 2000 mg/kg treatments with PS-UA can be seen in Figure 3.

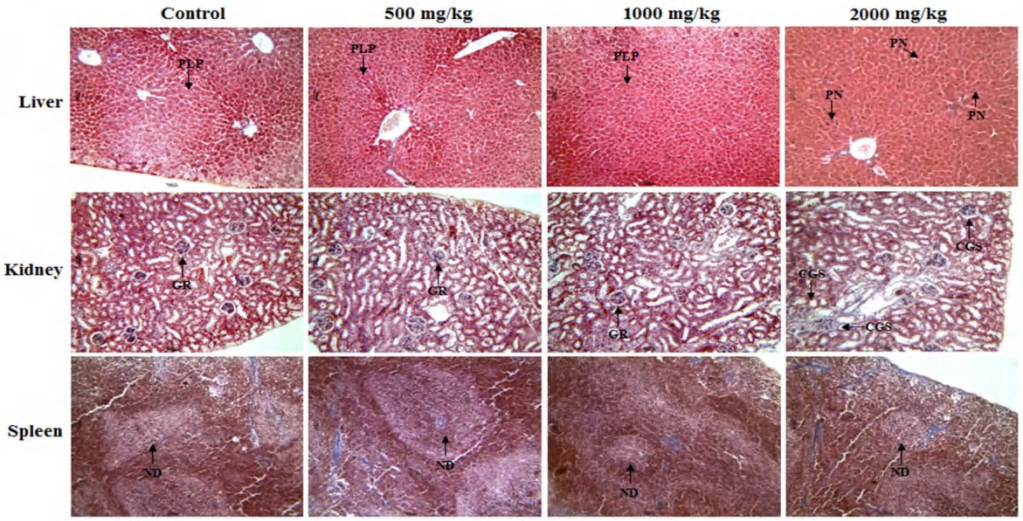

Figure 3. Representative photomicrographs of the livers, kidneys, and spleens of control mice (oral saline) and those treated with PS-UA (500, 1000, and 2000 mg/kg; oral administration). The liver with preserved liver parenchyma (PLP) and without significant histopathological changes compared with in the treatment of 2000 mg/kg, the liver presented pyknotic nucleus (PN). Kidneys: Renal glomeruli (GR) and contorted tubules without changes are visible in the control and groups treated with PS-UA at the 500 and 1000 mg/kg doses, while in the 2000 mg/kg treatment, the kidneys showed changes in the glomerular space, with morphological distortions. Spleen: The lymphatic nodes (ND) are well-defined in the control and treated groups. Hematoxylin and eosin staining was used. All images are with 100× magnification.

The normal architecture of hepatic, renal and splenic organs are observed in the control and in treatments with 500 and 1000 mg/kg of PS-UA. In the liver, the liver parenchyma was preserved and there were no significant histopathological changes. In the kidney, a glomerular architecture could be observed and Bowman's capsule and renal tubules were without inflammatory changes. In the spleen, splenic structure was preserved with well-defined lymphoid follicles in both treated and control groups. On the other hand, at the 2000 mg/kg dose histological alterations in the hepatic and renal tissues were observed. In the liver, the destructive fragmentation of the hepatocyte nucleus, a phenomenon called pynotic nucleus (PN) and characterized by the irregular distribution of chromatin from programmed cell death (apoptosis), was indicative of future tissue necrosis [48]. In the kidney there were changes in the glomerular space, with morphological distortions. As the kidney expands, it restricts and distorts the glomerular capillaries, decreasing the filtration surface of the capillary. These changes demonstrate why the mice treated with 2000 mg/kg consumed 50% less water than the control group. In this situation, the lesions may obliterate the glomerulus, with consequent reduction in renal function due to the lack of water intake. This was reported by a significant increase in creatinine value indicating renal change (Table 4). When these changes persist, it can lead to chronic or even terminal renal failure [48]. However, the splenic tissue did not present significant changes with defined and preserved lymph nodes.

Acute toxicity assay is usually conducted to determine the safety of a substance at determined dose ranges, but it can also provide initial information on toxicity mechanisms through the investigated parameters, mainly for the development of drugs with a pharmacological profile, whose therapeutic finality encompasses several diseases, mainly those ones that include pain, but showing less adverse effects and being effective at low concentrations.

2.5. Antinociceptive Activity

The antinociceptive activity of PS-UA was evaluated by two methods: acetic acid-induced contortion and the formalin test. The drug chosen was the reference indomethacin, where it showed an antinociceptive effect as expected. However, the concentrations with antinociceptive action (10 and 20 mg/kg of PS-UA) were associated to the doses that exhibited a macroscopic reduction of hepatic tissue, and hepatoprotector effect, after inhibition of hepatic metastasis of colorectal cancer in the murine model [21]. The treatments performed with doses of 10 and 20 mg/kg of PS-UA significantly inhibited ($p < 0.001$) the number of abdominal contortions induced by the intraperitoneal administration of acetic acid, when compared to the control group (Figure 4).

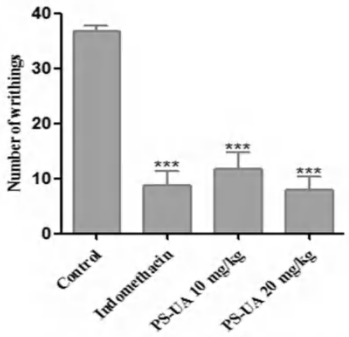

Figure 4. The antinociceptive effect of PS-UA (10 and 20 mg/kg; oral administration) and the reference drug indomethacin (20 mg/kg; intraperitoneal administration) in the acetic acid-induced writhing assay. The bars represent the mean numbers of writhing ± SD. (***) indicates a significant difference ($p < 0.001$) in the number of contortions versus the control.

The groups treated with either 10 and 20 mg/kg of PS-UA dose presented a reduction of 68% and 78%, respectively, but with no significant difference ($p > 0.5$) between the concentrations. The contortions induced by acetic acid are caused by the irritation of the acid injected intraperitoneally involve the stimulation of peripheral nociceptors and consequently causes behavioral reactions triggering the release of mediators such as P substances, bradykinins, prostaglandins and proinflammatory cytokines. These, in turn, stimulate peripheral nociceptors and neurons sensitive to inflammatory mediators [49]. Prostaglandin and bradykinin, for example, cause changes not only in specific receptors (TRPV1) coupled to the ion- and binder-dependent channels via cAMP activation but also in the protein kinases A (PKA) and C (PKC), reducing neural membrane post-hyperpolarization time and causing a reduction in the threshold for firing of the nerve fiber [50]. Regarding the antinociceptive activity of UA, Okuyama et al. [9] reported analgesic effects of acetic acid-induced contortion and tail pressure in mice for treatments of 30 and 100 mg/kg, where they concluded that acetic acid-induced pain was only decreased at a concentration of 100 mg/kg, while for tail pressure both treatments presented significant analgesic results. Therefore, it is believed that the potentiality of the analgesic activity of PS-UA by the acetic acid-induced contortion method in treatments of 10 and 20 mg/kg can be attributed to the K^+ radical, since the K^+ present in the structure of PS-UA is the only element that differentiates it from UA and confers hydrophilic characteristics to PS-UA, increasing its bioavailability and its pharmacological effects on pain.

In the formalin test, the antinociceptive activity of PS-UA was also detected in the 10 and 20 mg/kg treatments, acting very significantly ($p < 0.001$) in the neurogenic and inflammatory phases (Figure 5), and reducing the time mice spent licking their paw by 55% and 81% in the first phase (neurogenic pain) in the respective treatments. In the second phase (inflammatory pain), PS-UA showed an antinociceptive effect again at both doses, reducing the time of paw licking by 53% and 73%, respectively. On the other hand, indomethacin suppressed the response only in the second phase by 66%, while morphine was active in both phases corresponding to an 88% and 95% reduction, respectively. When PS-UA was associated with naloxone it suppressed the response of inflammatory pain only in the second phase for both treatments, corresponding to 72% and 86% respectively.

Naloxone is a non-selective opioid receptor antagonist that competes directly with morphine for catalytic sites/linkers. This suggests that the antinociceptive effect of PS-UA is mediated by the activation of opioid receptors, demonstrating a strong antinociceptive effect in neurogenic (non-inflammatory) pain and inflammatory pain [8,51].

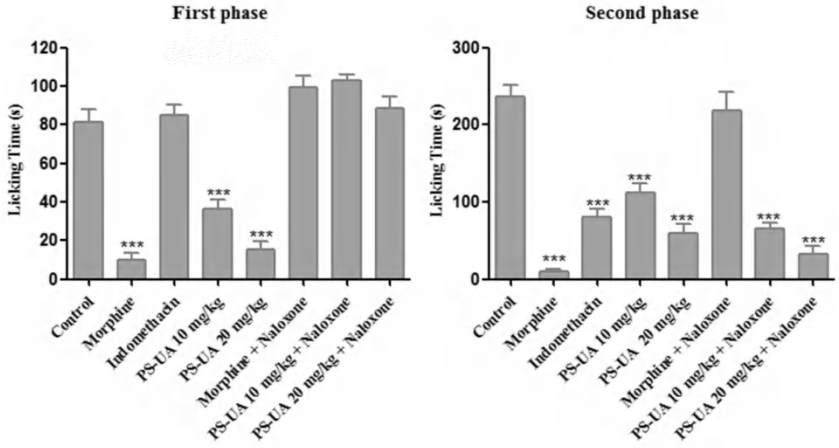

Figure 5. The antinociceptive effect of PS-UA (10 and 20 mg/kg; oral administration) and the reference drugs, indomethacin (20 mg/kg; intraperitoneal) and morphine (10 mg/kg; oral administration), in both phases of the formalin assay. The bars represent the mean time spent by the mice licking their paws ± SD. The involvement of opioid receptors in the antinociceptive effect was also evaluated by administering naloxone (5 mg/kg, intraperitoneal) to the mice 30 min before the administration of PS-UA or morphine. (***) indicates significant difference ($p < 0.001$) in the licking time versus the control.

Opioid and non-opioid analgesics are the mainstay of pain management. Antidepressants, anticonvulsants and other drugs active on the central nervous system can be used for chronic, neuropathic pain or even in the stages of terminal illnesses, being the first-line treatments for some conditions with the purpose of reducing pain and related incapacities [52]. The nociceptive drugs that act at the level of the central nervous system in the first phase are the main agents that act in the neurogenic phase of nociception and present an action mechanism characterized by the direct activation of the sensory C fibers through the cationic potential channel of the transient receptor, subfamily A, member 1 (TRPA1) [53–55].

In inflammatory pain (second phase), the nociceptors are sensitized by the action of chemical substances, called algiogenic agents, present in the tissue environment. This results in the release of the inflammatory mediators acetylcholine, bradykinin, histamine, serotonin, substance P, leukotriene, platelet activation factor, acid radicals, potassium ions, prostaglandins, thromboxane, interleukins, tumor necrosis factor (TNFα), nerve growth factor (NGF) and cyclic adenosine monophosphate (cAMP) [50]. An important finding is the comparison of the antinociceptive effect of morphine with the treatment at the 20 mg/kg dose of PS-UA in the first and second phases, where no significant difference ($p > 0.05$) was found between the two drugs. Thus, we can affirm from the results that PS-UA was effective in reducing local (first stage, neurogenic pain) and inflammatory (second phase) pain, interfering in the central nociceptive pathway, which corroborates its dual efficacy in both the acetic acid-induced abdominal contortions and the formalin test.

3. Material and Methods

3.1. Lichen Collection

The species *Cladonia substellata* Vanio (1887) was collected in February 2015 in the city of Mamanguape, Paraíba (PB-Brazil) 6°42′1.5″S 35°8′3.3″W. A voucher specimen was deposited in the

herbarium Geraldo Mariz, Department of Botany of the Federal University of Pernambuco (UFPE), Recife/PE, Brazil (voucher n° 77.474).

3.2. Extraction and Isolation of Usnic Acid and its Modification into Potassium Salt

C. substellata (120 g) was extracted with diethyl ether (5×) in a Soxhlet apparatus at 40 °C for 16 h. Each extraction was dried at room temperature (28 ± 3 °C). Subsequently, the extracts were filtered and concentrated to dryness on a rota-evaporator coupled to a water bath at 37 °C. UA was isolated and purified on a silica gel column (70–230 mesh) and eluted with chloroform-hexane (80:20 *v/v*). The fractions obtained were monitored by thin-layer chromatography (TLC), TLC: 0.1 mg samples of the fractions obtained were dissolved in acetone (0.5 mL). Then, 1 µL of the solution was applied to a silica gel plate (Gel 60 F254+366 Merck®, Darmstadt, Germany) measuring 20 × 20 cm. TLC assays were carried out under increasing polarity conditions using solvent system A (toluene/dioxane/acetic acid, 36:9:1, *v/v/v*) for the UA. The spots were observed under ultraviolet light (256–366 nm) and visualized on the plate (Fisatom model 509, São Paulo, Brazil), after spraying of 10% sulfuric acid, and heated at 50 °C for 20 min. The composition was evaluated by the determination of the retention factor (Rf) and comparison with the standard single acid [23]. High-performance liquid chromatography (HPLC), HPLC: A Hitachi chromatograph (655 A-11, Tokyo, Japan) was coupled to a UV detector (655 A-11, Tokyo, Japan) at 254 nm and a reverse phase column (RP 18 MicroPack MCH-18, 300-4 mm, Berlin, Germany). The UA was dissolved in diethyl ether (Merk® KGaA, Darmstadt, Germany) at a concentration of 1.0 mg mL^{-1} and injected. The mobile phase consisted of methanol/deionized water/acetic acid (80:19.5:0.5 *v/v/v*) with a flow of 1.0 mL min^{-1}, 0.04 attenuation at room temperature (28 ± 3 °C). The substance was identified based on its retention time (RT) and peak area when compared to standard UA [23]. The molecular structure was determined by proton nuclear magnetic resonance (^{1}H-NMR) and carbon (^{13}C-NMR) obtained at 400 MHz in acetone d-6 (Varian UNITY spectrometer, Santa Clara, CA, USA), while infrared spectroscopy (IR) analyses were performed in a Bruker Fourier spectrometer (model IFS 66, Ettlingen, Germany) with KBr disks [23,25]. In addition, the optical rotation of UA was determined in a Jasco P2000 polarimeter (Jasco Incorporated, Easton, MD, USA) at the Analytical Centre of Fundamental Chemistry Department of Federal University of Pernambuco.

After chemical characterization and confirmation of the purity of UA, 1 g of the compound was added to 800 mL of water milli-q, and 5 mL KOH (5%) solution was gently dropped until complete solubilization of UA, until pH 11 was reached. The solution was frozen at −80 °C, lyophilized (reaction that give 100% yield), and stored in desiccator. The confirmation of the PS-UA structure was done through ^{1}H-NMR, and IR and elemental analysis (C, H) was performed using a Perkin Elmer CHN-2400 (Waltham, MA, USA) at University of São Paulo–USP [23,25].

3.3. Computational Methods

All the calculations where performed using the Gaussian 09 program (Gaussian Inc., Wallingford, CT, USA) [28]. The geometry optimization of tautomer UA, which is the most stable according to previous theoretical studies [29,30], was performed with semi-empirical method AM1, and the electronic part of equilibrium geometries was obtained in accordance with the ab initio (DFT—density functional theory) level of theory, with the combination method/basis set being B3LYP/6-31g+(d,p). The same procedure was applied to the anions of UA, formed after each possible deprotonation on carbons 1, 2 or 3 (A1, A2, and A3, respectively), with or without a previous proton abstraction. The energies in the aqueous solution were obtained in accordance with the SMD (solvent model density) implicit solvent model, from the ideal gas equilibrium geometries [56]. Calculation of ESP charges of UA atoms was performed in accordance with the Breneman and Wiberg algorithm implemented by the input line (POP=CHELPG) [57], in both ideal gas and water.

3.4. Acute Toxicity Evaluation

Acute toxicity experiments were carried out using Swiss webster mice (32 ± 2 g) reared at the Keizo Assami Immunopathology Laboratory (LIKA) of UFPE and kept under a controlled environment (20 ± 2 °C, 12 h light/dark cycle) (libitum/Purina, São Paulo (SP)). All experimental procedures were only carried out after approval by the Animal Experimentation Ethics Committee (CEEA) of the Bioscience Center, Federal University of Pernambuco (UFPE) (Process Nº. 23076.015163/2017-65).

The acute toxicity effect of PS-UA was performed according to the guidelines of the Organization for Economic Cooperation and Development [36]. Acute toxicity (mortality and behavioral changes) was evaluated by oral administration. PS-UA was dissolved in distilled water to avoid the effect of salting-out. Mice were divided into 4 groups (n = 5): a control group which received filtered water and three groups that were treated with PS-UA at doses of 500, 1000 and 2000 mg/kg. Mice were observed for 5 days. On the first day, behavioral changes were observed every 10 min for 4 h, followed by two observations one day after administration in order to record toxicity-related behaviorial signs through the parameters: respiratory frequency, piloerection, stereotyped movement, fine tremors, lifting upper train, spasms, prostation, lowering hind quarters, photophobia, fecal excretions, abdominal distension, and death [58]. From the time of treatment, variations in body weight were determined, as well as the daily consumption of water and food. On the 5th day after the start of treatment, peripheral blood was collected, then the mice were euthanized, and the liver, kidney and spleen were removed, weighed macroscopically and processed for histological evaluation.

3.5. Haematological and Biochemical Analyses

The blood collected was used to evaluate hematological and biochemical alterations. The following hematologic parameters were analyzed using an automatic analyzer (CELL-DYN Ruby, Lake Bluff, IL, USA) and optical microscopy (Olympus BX 41, Olympus Corporation of the Americas, Center Valley, PA, USA.): erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and total and differentiated analyses of leukocytes. For the biochemical analysis, blood was evaluated for albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, total cholesterol, triglycerides, urea, and creatinine using specific kits (Labtest Diagnóstica, Lagoa Santa, Brazil).

3.5. Histopathological Analysis

Histological analyses of the liver, kidney, and spleen of the control and PS-UA-treated animals were performed by optical microscopy. Fragments of the organs were fixed in buffered formalin (10% *v/v*), then dehydrated through a graduated series of ethanol (70, 80, 90, and 100%), diaphonized with xylol, and embedded in paraffin. Histological sections (5 μm thick) were stained with hematoxylin-eosin and fitted under coverslips with Entellan resin (Merck, Darmstadt, Germany). The slides were observed under a Labomed Lx400 microscope coupled to a Moticam 1000 digital camera of 1.3 MP USB 2.0 using Motic Images Plus 2.0 software (Motic Incorporation Ltd., Hong Kong, China).

3.6. Evaluation of Antinociceptive Activity

3.6.1. Acetic Acid-Induced Writhing Test

Female mice were separated into four groups (n = 6): Group I treated with oral saline (control), Group II intraperitoneal indomethacin (20 mg/kg), and Groups III and IV with PS-UA at concentrations of 10 and 20 mg/kg, respectively. Saline or PS-UA were administered via an esophageal catheter 1 h prior to acetic acid administration, while indomethacin was given 30 min before. Each animal received an intraperitoneal injection of 0.85% (*v/v*) acetic acid in saline and was then placed in a polyethylene box to record the latency period (time until the first writhing) and the number of writhes in the interval corresponding to 5–15 min after the injection of acetic acid (CEEA process Nº. 23076.015163/2017-65)[59].

3.6.2. Formalin Test

The procedure used was similar to that described by Ping et al. [60]. Female mice were divided into 8 groups ($n = 6$) and the following pretreatments were administered: Group I Vehicle (100 µL of water, v.o.); Group II Morphine (10 mg/kg); Group III Indomethacin (20 mg/kg); and Groups IV and V PS-UA 10 and 20 mg/kg, respectively (v.o.). All animals were treated through gavage. After 60 min, 20 µL of 2.5% (*v*/*v*) formalin in saline was injected into the subplantar region of the right hind paw of each animal. The time spent by the mouse licking its paw was recorded during the first 5 min after formalin injection (first phase neurogenic pain), as well as 15–30 min after the injection (second phase inflammatory pain). In order to evaluate the involvement of opioid receptors and the PS-UA action mechanism, naloxone (5 mg/kg, intraperitoneal) was administered 30 min earlier, morphine 10 mg/kg + naloxone for Group VI, and 10 and 20 mg/kg + naloxone for Groups VII and VIII PS-UA, respectively [61].

3.7. Statistical Analysis

The results are expressed as the means of replicates ± standard deviation (SD). Analysis of variance (ANOVA) was performed followed by Tukey's test for multiple comparisons. A p value < 0.05 was adopted as the significance level.

4. Conclusion

PS-UA presented moderate oral toxicity to mice, since no animal death at a dose of 1000 mg/kg was detected. However, PS-UA promoted hematological, biochemical, and histopathological changes and death at the concentration of 2000 mg/kg. The 500 mg/kg treatment of PS-UA has been shown to be an interesting dose for future pharmacological investigations without toxic effects. The results also show that PS-UA had antinociceptive activity and was active against both noninflammatory and inflammatory pain at the concentrations 10 and 20 mg/kg, being associated with interference of the opioid receptor pathway. This work contributes to the knowledge of the acute toxicity of PS-UA and to its pharmacological action for pain.

Supplementary Materials: The Supplementary Materials are available online.

Author Contributions: H.D.A.A., J.G.S.J., M.C.B.M., A.L.A., E.C.P., and V.L.M.L. designed the study protocol. H.D.A.A., J.G.S.J., J.R.S.O., M.H.M.L.R., M.C.B.M., M.A.C.B., A.L.A., M.C.P.A.A., N.T.P.F., M.R.M.-J., E.C.P., D.J.R.S., J.V.A., E.P.S.F., N.H.S., and V.L.M.L. carried out the assays and were involved in the analysis and interpretation of all data. H.D.A.A., J.G.S.J., M.C.B.M., A.L.A., M.R.M.-J., E.C.P., D.J.R.S., J.V.A., E.P.S.F., N.H.S., and V.L.M.L. contributed to drafting the manuscript and/or critically revising the paper and intellectual content. All authors read and approved the final manuscript.

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Sample Availability: Samples of the compounds are not available from the authors.

4. CONCLUSÕES

Os resultados deste estudo demonstraram efeitos tóxicos e teratogênicos do sal de potássio do ácido úsnico (SP-AU) nos estágios embrionários do molusco *B. glabrata*. Esta substância também foi eficiente no combate às cercárias do *S. mansoni*. Portanto, sugere-se que o SP-AU possa ser utilizado como alternativa no controle populacional do hospedeiro intermidiário e sobre a forma infecciosa do patógeno em concentrações ambientalmente seguras.

Em relação aos resultados da avaliação esquistossomicida, o SP-AU apresentou uma maior eficácia sobre os estágios evolutivos do *S. mansoni* do que ao praziquantel (PZQ), medicamento adotado pela Organização Mundial da Saúde. Além disso, os resultados obtidos pela microscopia eletrônica de varredura demonstraram alterações ultraestruturais mas pronunciadas nos tegumentos dos vermes expostos ao PS-UA, quando comparado ao PZQ.

O SP-AU apresentou toxicidade oral moderada em camundongos, uma vez que nenhuma morte de animal na concentração de 1000 mg/kg foi observada. No entanto, o SP-AU promoveu alterações hematológicas, bioquímicas, histopatológicas e morte na concentração de 2000 mg/kg. A concentração de 500 mg/kg do SP-AU demonstrou ser uma dose interessante para futuras investigações farmacológicas envolvendo doenças tropicais e negligenciadas em modelo *in vivo* uma vez que não apresentou efeitos tóxicos.

Portanto, considerando a necessidade urgente de novos moluscicidas e fármacos esquistossomicidas o os efeitos apresentados pelo SP-AU, essa molécula pode representar um novo composto para o futuro controle/combate da esquistossomose mansônica.

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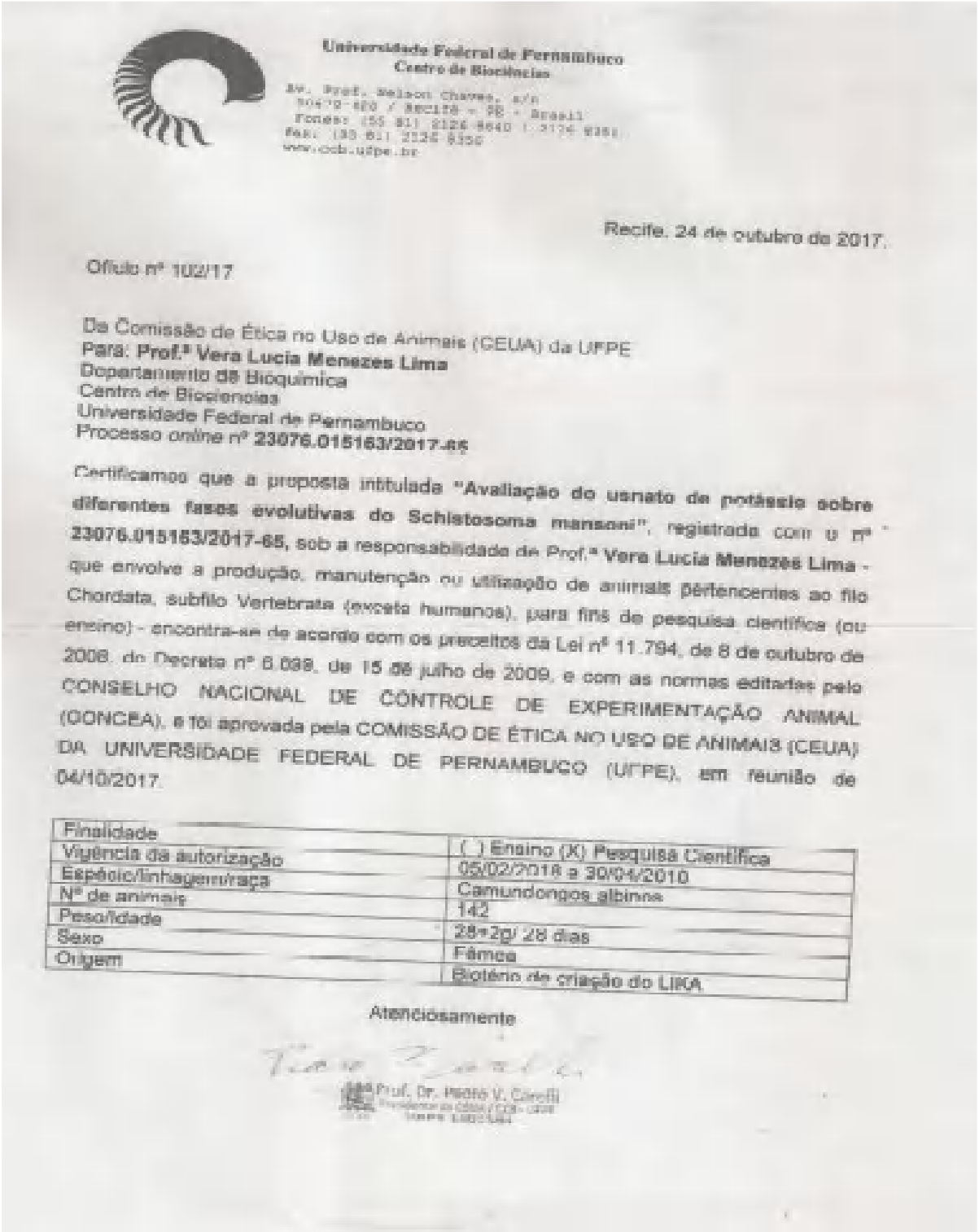
ANEXO A – COMITÊ DE ÉTICA NO USO DE ANIMAIS



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Recife, 24 de outubro de 2017.

Ofício nº 102/17

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof.ª Vera Lucia Menezes Lima**
Departamento de Bioquímica
Centro de Biociências
Universidade Federal de Pernambuco
Processo *online* nº **23076.015163/2017-65**

Certificamos que a proposta intitulada **"Avaliação do usnato de potássio sobre diferentes fases evolutivas do Schistosoma mansoni"**, registrada com o nº **23076.015163/2017-65**, sob a responsabilidade de Prof.ª **Vera Lucia Menezes Lima** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - 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 PERNAMBUCO (UFPE), em reunião de 04/10/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	05/02/2018 a 30/04/2010
Espécie/linhagem/raça	Camundongos albinos
Nº de animais	142
Peso/Idade	28±2g/ 28 dias
Sexo	Fêmea
Origem	Biotério de criação do LIKA

Atenciosamente

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
SIAPE 1380144

ANEXO B - LINK DO GUIA DOS AUTORES DO PERIÓDICO ACTA TROPICA EDITORIAL ELSEVIER

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