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HIANNA ARELY MILCA FAGUNDES SILVA

AVALIAÇÃO DA SUSCEPTIBILIDADE *IN VITRO* DE *Schistosoma mansoni* E DE *Biomphalaria glabrata* FRENTE A COMPOSTOS LIQUÊNICOS

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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutora em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

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Orientador: Prof. Dr. Nicácio Henrique da Silva

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RESUMO

A esquistossomose é uma doença parasitária que afeta cerca de 260 milhões de pessoas no mundo. Atualmente apenas o Praziquantel é utilizado para o tratamento de indivíduos infectados, havendo relatos do surgimento de cepas tolerantes ao medicamento. Para o controle de vetores da esquistossomose, a Niclosamida é o único moluscicida recomendado, entretanto, apesar de eficaz, é tóxico para o meio ambiente, possui alto custo e se decompõe sob raios UV. Este trabalho teve como objetivo: (1) avaliar a atividade do extrato etéreo do liquen *Ramalina aspera* sobre embriões e caramujos adultos da espécie *Biomphalaria glabrata* e cercárias de *Schistosoma mansoni* e (2) analisar as atividades esquistossomicidas *in vitro* dos metabólitos secundários liquênicos, ácidos barbático e divaricático, extraídos de *Cladia aggregata* e *Canoparmelia texana*, respectivamente, sobre vermes adultos de *S. mansoni*. O extrato etéreo de *R. aspera* demonstrou atividade moluscicida tanto para embriões (LC_{90} de 22.78, 24.23, 16.63 e 16.03 µg mL^{-1} para as fases de gástrula, blástula, trocófora e véliger, respectivamente), como para caramujos adultos (LC_{90} de 8.66 µg mL^{-1}). As doses subletais causaram a diminuição da fertilidade em 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^{-1}, não demonstrando toxicidade por meio do bioensaio com *Artemia salina.* Na avaliação esquistossomicida *in vitro* dos ácidos barbático e divaricático, foi observada atividade dos compostos a partir de 3 h de exposição. Ao final de 24 h, na avaliação de motilidade, o ácido barbático apresentou letalidade sobre *S. mansoni* nas concentrações de 50 - 200 µM e o ácido divaricático nas concentrações de 100 e 200 µM. Alterações de motilidade foram observadas nas concentrações subletais para ambos os ácidos. A IC_{50} obtida no ensaio de viabilidade celular dos vermes adultos, através da metodologia por MTT, foi de 100.6 µM para o ácido divaricático e 99.43 µM para o ácido barbático. Danos extensivos ao tegumento dos vermes foram causados pelos ácidos divaricático e barbático como descamação, erosão, formação de bolhas, edema e danos tuberculares. A citotoxicidade dos ácidos em células humanas foi avaliada por meio do ensaio com células mononucleares do sangue periférico (PBMCs), onde os resultados demonstraram atoxicidade até a concentração de 200 µM. Pode-se concluir que o extrato de *R. aspera* demonstrou promissora atividade moluscicida, visando o controle de *B. glabrata*, assim como atividade cercaricida e baixa toxicidade sobre *A. salina*. O efeito esquistossomicida *in vitro* dos ácidos barbático e divaricático sobre *S. mansoni* foi comprovado, causando morte, alterações de motilidade e

danos ultraestruturais aos vermes, em concentrações que não provocaram citotoxicidade sobre PBMCs.

Palavras-chave: Líquens. Atividade esquistossomicida. Atividade moluscicida. *Schistosoma mansoni*. *Biomphalaria glabrata*.

ABSTRACT

Schistosomiasis is a parasitic disease that affects about 260 million people worldwide. Currently only Praziquantel is used to treat infected individuals, with reports of drug-tolerant strains. For the control of schistosomiasis vectors, Niclosamide is the only recommended molluscicide. However, although effective, it is toxic to the environment, has a high cost and decomposes under UV rays. The objective of this study was: (1) to evaluate the activity of ether extract of lichen *Ramalina aspera* on embryos and adult snails of the species *Biomphalaria glabrata* and *Schistosoma mansoni* cercariae and (2) to analyze the *in vitro* schistosomicidal activities of lichen secondary metabolites, barbatic and divaricatic acids, extracted from *Cladia aggregata* and *Canoparmelia texana*, respectively, on adult *S. mansoni* worms. The *R. aspera* ether extract showed molluscicidal activity for both embryos (LC_{90} of 22.78, 24.23, 16.63 and 16.03 µg mL^{-1} for gastrula, blastula, trocophore and veliger phases, respectively) and for adult snails (LC_{90} of 8.66 µg mL^{-1}). Sublethal doses caused decreased fecundity in adult snails and quantitative and morphological changes in their hemocytes. The ether extract of *R. aspera* also exhibited cercaricidal effect from 5.0 µg mL^{-1}, showing no toxicity by *Artemia salina* bioassay. In the *in vitro* schistosomicidal evaluation of the barbatic and divaricactic acids, activity of the compounds was observed after 3 h of exposure. At the end of 24 h, in the motility evaluation, the barbatic acid presented lethality over *S. mansoni* at 50 - 200 µM and the divaricatic acid at 100 and 200 µM. Motility changes were observed in sublethal concentrations for both acids. The IC_{50} obtained in the cell viability assay of adult worms using the MTT methodology was 100.6 µM for divaricatic acid and 99.43 µM for barbatic acid. Extensive damage to the tegument of the worms was caused by the divaricatic and barbatic acids such as peeling, erosion, blistering, edema and tubercular damage. The cytotoxicity of lichen acids in human cells was assessed by the peripheral blood mononuclear cell (PBMCs) assay, where the results demonstrated atoxicity up to 200 µM concentration. It can be concluded that *R. aspera* extract showed promising molluscicidal activity aiming at controlling *B. glabrata*, as well as cercaricidal activity and low toxicity on *A. salina*. The *in vitro* schistosomicidal effect of barbatic and divaricatic acids on *S. mansoni* has been proven to cause death, motility changes and ultrastructural damage to worms at concentrations that did not cause cytotoxicity on PBMCs.

Keywords: Lichens. Schistosomicidal activity. Molluscicidal activity. *Schistosoma mansoni*. *Biomphalaria glabrata.*

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LISTA DE ABREVIATURAS

TLC	*Thin-layer chromatography*
CL	*Confidence limit*
HPLC	*High performance liquid chromatography*
DMSO	*Dimethyl sulfoxide*
DMSO-D6	*Dimethyl sulfoxide deuterated*
EDTA	*Ethylenediaminetetraacetic acid*
IC	*Inhibitory concentration*
IR	*Infrared spectroscopy*
LC	*Lethal concentration*
SEM	*Scanning electron microscopy*
MTT	*Thialzolyl blue tetrazolium bromide*
OMS (WHO)	Organização Mundial de Saúde (*World Health Organization*)
PBMC	*Peripheral blood mononuclear cells*
PBS	*Phosphate buffered saline*
PZQ	Praziquantel
^{13}C NMR	*Carbon nuclear magnetic resonance*
^{1}H NMR	*Proton nuclear magnetic resonance*
RT	*Retention time*
SD	*Deviation standard*
SEA	*Soluble egg antigens*
UV	Ultravioleta

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1 INTRODUÇÃO

A esquistossomose é considerada uma doença de grande impacto social, ~~e~~ representa um sério problema de saúde pública em diversos países tropicais e subtropicais. Causada por vermes trematódeos do gênero *Schistosoma*, a esquistossomose tem prevalência em comunidades que vivem sob condições inadequadas de saneamento e sem acesso à água potável, levando indivíduos infectados a desenvolverem uma série de complicações clínicas que, em casos mais graves, podem culminar em óbito (CADEDOS, 2018).

É estimado que, no mundo, cerca de 261 milhões de pessoas estejam infectadas por vermes *Schistosoma*, havendo em torno de 200.000 óbitos anuais, e, aproximadamente, 700 milhões de indivíduos vivam em áreas endêmicas onde há o risco de contrair a doença (WHO, 2015; FEITOSA et al., 2018; EISSA; EL BARDICY; TADROS, 2011). Na África e Américas Central e Sul, a espécie *S. mansoni* é a responsável por causar a esquistossomose intestinal, tendo como hospedeiro intermediário caramujos do gênero *Biomphalaria* (COLLEY et al., 2014).

A interrupção do ciclo de vida do parasita é essencial para impedir a transmissão da doença. Além do tratamento quimioterápico de indivíduos infectados, faz-se necessária a utilização de meios alternativos de interrupção da transmissão através da redução de hospedeiros intermediários em locais endêmicos e a implementação de saneamento básico para evitar a contaminação hídrica (KING; BERTSCH, 2015).

Atualmente, não existe uma vacina eficaz implantada contra a esquistossomose, e o tratamento com o praziquantel é a única forma eficaz de controlar a infecção pelo *Schistosoma* (LORSUWANNARAT et al., 2013). O praziquantel, hoje, é a droga de escolha contra o parasito, que atua induzindo contrações espamódicas sobre o corpo do helminto através do influxo de Ca^{++} pela membrana celular (BECKER et al.,1980). Entretanto, utilização da quimioterapia massiva com o praziquantel vem causando preocupação devido ao surgimento de resistência/tolerância em residentes de áreas endêmicas que recebem tratamento sequencial com o praziquantel (ABDUL-GHANI et al., 2009).

No que concerne ao controle dos caramujos vetores, atualmente, o único moluscicida recomendado pela Organização Mundial de Saúde é a Niclosamida (Bayluscide, Bayer) (WHO, 2002), substância sintética, que apesar de eficiente no controle dos caramujos vetores, apresenta alto custo em sua aplicação, se decompõe sob luz solar e apresenta elevado nível de toxicidade para organismos não-alvo, como peixes, anfíbios e plantas (OLIVEIRA-FILHO; PAUMGARTTEN, 2000; ABREU; GOULART; OLIVEIRA BRETT, 2002; GRAEBING et

al., 2004; FARIA et al., 2018). A procura por moluscicidas de origem natural, não tóxicos ao meio ambiente e eficazes no combate aos moluscos vetores e à fase larval infectante do parasita (cercárias) se faz necessária, surgindo como alternativa, principalmente em áreas rurais de países em desenvolvimento, onde a doença é endêmica (LIMA et al., 2002; SHENG-XIA et al., 2007; KE et al., 2017).

O foco da busca por novos compostos para o controle da esquistossomose se intensificou sobre os produtos de origem natural, sendo observados na literatura diversos trabalhos que reportam o estudo da atividade anti-helmíntica e moluscicida de produtos naturais (MORAES et al., 2012; CASTRO et al., 2015; ROCHA-FILHO et al., 2015; MARTINS et al., 2017; ARAÚJO et al., 2018).

Líquens, associações simbióticas entre fungos e algas, produzem substâncias que se classificam em produtos intracelulares (proteínas, carboidratos, vitaminas e carotenóides) e extracelulares (metabólitos secundários de natureza fenólica). Os diversos metabólitos produzidos pelos líquens, sendo a grande maioria de natureza fenólica, são organizados em diferentes classes, como as classes dos depsídeos, depsidonas, dibenzofuranos, xantonas, ésteres benzílicos, antraquinonas, dentre outras (HONDA; VILEGAS, 1998; BELLIO et al., 2015).

Na literatura, encontram-se relatos da utilização de materiais ricos em compostos fenólicos com propriedades antibacterianas, antitumorais, antinflamatórias, fungicidas, antivirais, moluscicidas e anti-helmínticas (PUUPPONEN-PIMIÄ et al., 2001; MILES et al., 2005; ARAÚJO, 2009; DAI; MUMPER, 2010; HU et al., 2013; LORSUWANNARAT et al., 2013; ZABKA; PAVELA, 2013; MARTINS et al., 2017;). Neste contexto, os extratos liquênicos e seus metabólitos fenólicos se mostram promissores em avaliações esquistossomicidas e moluscicidas, visando uma alternativa para o tratamento e controle da esquistossomose.

Diante do exposto, e levando-se em consideração o extenso potencial biológico das substâncias liquênicas, este trabalho apresenta uma análise das atividades moluscicida, cercaricida e esquistossomicida dos metabólitos liquênicos oriundos dos líquens *Ramalina aspera*, *Canoparmelia texana* e *Cladia aggregata*.

1.1. OBJETIVOS

1.1.1 Objetivo geral

- Avaliar a atividade moluscicida, cercaricida e artemicida do extrato etéreo de *Ramalina aspera* sobre *Biomphalaria glabrata*, *Schistosoma mansoni* e *Artemia salina*, e a atividade esquistossomicida *in vitro* e citotóxica dos ácidos divaricático e barbático, extraídos de *Canoparmelia texana* e *Cladia aggregata*, sobre *Schistosoma mansoni* e células mononucleares de sangue periférico.

1.1.2 Objetivos específicos

- Obter os extratos etéreos de *Ramalina aspera*, *Canoparmelia texana* e *Cladia aggregata*;
- Isolar e caracterizar os ácidos divaricático e barbático a partir dos líquens *C. texana* e *C. aggregata*, respectivamente.

ARTIGO 1

- Avaliar a atividade moluscicida do extrato etéreo de *R. aspera* e seus efeitos sobre a reprodução de moluscos adultos de *Biomphalaria glabrata;*
- Avaliar morfologicamente e quantitativamente os hemócitos de *B. glabrata* após exposição do molusco ao extrato etéreo de *R. aspera*;
- Verificar a atividade embriotóxica do extrato etéreo de *R. aspera* sobre *Biomphalaria glabrata* em diferentes fases embrionárias;
- Avaliar a atividade cercaricida do extrato etéreo de *R. aspera* sobre *Schistosoma mansoni*.
- Analisar a toxicidade ambiental do extrato etéreo de *R. aspera* através do bioensaio utilizando *Artemia salina*.

ARTIGOS 2 E 3

- Avaliar a susceptibilidade *in vitro* de casais de vermes adultos de *S. mansoni* aos ácidos divaricático e barbático;

- Analisar a viabilidade celular de casais de *S. mansoni* através do método Thialzolyl Blue Tetrazolium Bromide (MTT)
- Avaliar alterações tegumentares nos vermes expostos aos ácidos divaricático e barbáticoatravés da microscopia eletrônica de varredura (MEV);
- Mensurar o efeito citotóxico dos ácidos divaricático e barbático sobre células mononucleares de sangue periférico (PBMC) humano.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 ESQUISTOSSOMOSE, UM BREVE HISTÓRICO

A esquistossomose é uma doença infecciosa parasitária causada por vermes trematódeos do gênero *Schistosoma* (COLLEY, 2014). Considerada uma doença antiga, seus aspectos clínicos foram descritos pela primeira vez pelo japonês Fuji, em 1857. Registros de Ruffer, no ano de 1910, relatam a descoberta de ovos de *Schistosoma* calcificados em múmias egípcias datadas por volta de 1200 a.C (PARAENSE, 2008; LEWIS; TUCKER, 2014). Através da detecção de antígenos circulantes do parasito nos tecidos de múmias egípcias, sabe-se hoje que a humanidade convive com a esquistossomose desde os 3000 a.C. (MILLER et al., 1992).

Na África, são identificadas um grande número de espécies de *Schistosoma*, por isso é razoável que muitos imaginem que a origem da espécie se deu no continente africano, se espalhando posteriormente para outros continentes (DAVIS, 1980, 1992). No entanto, estudos filogenéticos moleculares e estudos de padrões cromossômicos de bandas C revelaram que o *S. japonicum* possui uma posição basal dentro da árvore filogenética do gênero. Assim sendo, apesar de muitas espécies do gênero *Schistosoma* serem majoritariamente encontradas na África e do seu maior significado médico e veterinário, diferentes linhas de pesquisa sugerem que suas origens estão na Ásia (LOCKYER et al., 2003; NAHUM; MOURÃO; OLIVEIRA, 2012).

Em 1851, o médico Theodor Bilharz, descreveu pela primeira vez a doença em humanos após se deparar, em uma necropsia, com um helminto na veia porta de um jovem egípcio, dando o nome de *Distomum haematobium* ao verme e, posteriormente, *Schistosoma haematobium*. Como foi o primeiro pesquisador a descrever o parasito em detalhes, a esquistossomose também ficou conhecida como bilharzíase ou bilharziose. Quarenta anos depois, o médico Patrick Manson levantou a hipótese da existência de duas espécies de *Schistosoma* parasitas do homem. Hoje, sabe-se que 5 espécies são responsáveis pela doença em todo o mundo (KATZ; ALMEIDA, 2003).

A chegada e instalação da esquistossomose no Brasil se deu pela imigração de escravos africanos infectados, e a presença de hospedeiros intermediários do ciclo do *S. mansoni*. Os focos primários da doença se deram na região canavieira nordestina. A partir daí, com os movimentos migratórios (ciclo do ouro, ciclo da borracha, ciclo do café e industrialização), a esquistossomose se expandiu para outras regiões do país (NEVES, 2005).

A espécie existente no Brasil foi relatada pela primeira vez em 1907 pelo inglês Louis Sambon, que se baseou na observação de ovos característicos do trematódeo que continham espículo lateral, nomeando-a de *Schistosoma mansoni* em homenagem a Manson. No ano seguinte, Manoel Augusto Pirajá da Silva, médico brasileiro, publicou o primeiro trabalho sobre a doença no Brasil, intitulado "Contribuição para o estudo da Schistosomíase na Bahia". Em dois anos de estudos (1908-1909), Pirajá da Silva, pela primera vez, descreveu com riqueza de detalhes a espécie *Schistosoma mansoni*, já que as observações feitas por Sambon foram realizadas de forma superficial com apenas um representante macho da espécie. Foram três trabalhos publicados, onde Pirajá da Silva descreve 20 casos diagnosticados através de exames de fezes e autópsias. Foi por meio das preciosas observações de Pirajá da Silva que as incertezas taxonômicas da descrição de Sambon foram suprimidas, sendo decisivas para a validação da nova espécie (KATZ; ALMEIDA, 2003; KATZ, 2008; MATI, 2009; NOYA et al., 2015).

2.2 EPIDEMIOLOGIA DA ESQUISTOSSOMOSE

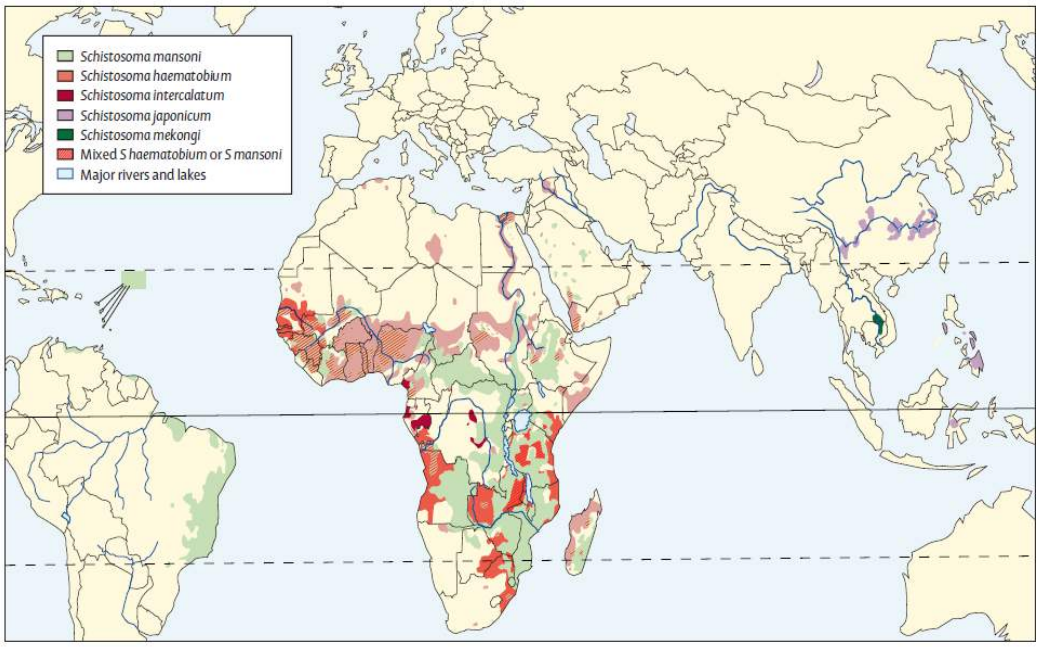
A esquistossomose é uma doença conhecida desde a Antiguidade e, a partir de uma perspectiva de saúde pública global, é a mais importante doença infecciosa com transmissão hídrica do mundo (STEINMANN et al., 2006; UTZINGER et al., 2009). É enquadrada na lista das Doenças Tropicais Negligenciadas (NTD – Neglected Tropical Disease) estabelecida pela Organização Mundial de Saúde (OMS), sendo estas um grupo diversificado de doenças que prevalecem em condições tropicais e subtropicais em 149 países, afetando mais de 1 bilhão de pessoas e matando cerca de 1 milhão de pessoas anualmente (WHO, 2009). O número de pessoas afetadas por estas enfermidades é mais elevado em regiões de maior pobreza, existindo uma relação direta entre a prevalência destas doenças e um baixo índice de desenvolvimento humano (IDH). Além disso, a infecção pelos agentes causadores de NTDs causa incapacidade e desfiguração, com profundo impacto econômico, social e político (LINDOSO; LINDOSO, 2009).

Em todo o mundo, aproximadamente 261 milhões de pessoas se encontram infectadas por vermes do gênero *Schistosoma* e mais de 700 milhões se encontram em situação de risco de contrair a doença (WHO, 2015; FEITOSA et al., 2018). Entretanto, a subestimação da verdadeira prevalência da esquistossomose tem sido relatada, devido, principalmente, à baixa sensibilidade da metodologia diagnóstica atualmente utilizada. Existe uma estimativa

afirmando que a prevalência da esquistossomose no mundo atingiu mais de 400 milhões de infectados (LIN et al., 2008; KING, 2010; SOUZA et al., 2017; KING; GALVANI, 2018).

Com prevalências em regiões tropicais e subtropicais, a esquistossomose é considerada endêmica em 78 países e territórios distribuídos entre a África, Ásia e Américas Central e Sul. Na Figura 1 é demonstrada a distribuição global da esquistossomose e das cinco espécies do gênero *Schistosoma* que apresentam importância médica na epidemiologia humana: *S. haematobium* (África e Oriente Médio), *S. intercalatum* (África Central), *S. japonicum* (China, Japão e Filipinas), *S. mekongi* (Sudeste Asiático) e *S. mansoni* (África, Antilhas, América Central e Sul) (COLLEY et al., 2014, WHO, 2019).

Figura 1 – Distribuição global da esquistossomose humana.



Fonte: COLLEY et al., 2014

A África responde por 97% de todas as infecções mundiais e 87% da população africana se encontra sob risco de infecção (STEINMANN et al., 2006; TLAMÇANI; ER-RAMI, 2014; UMAR et al., 2017). A prevalência da esquistossomose na África Subsaariana é considerada alta, sendo crianças e adultos jovens os mais acometidos pela doença. As maiores prevalências são encontradas na Nigéria, que é seguida pela República Unida da Tanzânia, Gana, República Democrática do Congo e Moçambique, que compõem os cinco principais países endêmicos da África (ADENOWO et al., 2015).

Na Ásia, os principais focos endêmicos da esquistossomose humana são encontrados na China, nas Filipinas e em pequenos bolsões da Indonésia (*S. japonicum*) e, em menor grau,

ao longo do rio Mekong, nas fronteiras do Camboja e do Laos (*S. mekongi*). No Japão, a eliminação da doença foi alcançada por meio do controle ambiental, ou seja, recuperação de terras para melhorar a produção agrícola e cimentação de valas usadas para a irrigação do arroz, e também através do desenvolvimento econômico social (ROSS et al., 2013).

Desde 1950, a China desenvolve um eficiente programa de controle da esquistossomose, onde o foco se volta para a eliminação de caramujos infectados. Dos 454 municípios endêmicos, 274 erradicaram completamente a ocorrência de transmissão (XU et al., 2015). Na Martinica e Arábia Saudita (*S. mansoni*) e Tunísia e Maurício (*S. haematobium*) esta patologia também foi controlada com sucesso ou mesmo eliminadas usando medidas integradas, que além a administração de medicamentos, levaram em consideração fatores ambientais, como a distribuição de hospedeiros intermediários, o auxílio de estratégias de engenharia preventiva e análises interrelacionadas de demografia, saúde e sistemas sociais. O Marrocos e alguns países caribenhos tem feito progressos significativos no controle da esquistossomose, enquanto o Brasil, China e Egito caminham na busca de alternativas para o controle desta doença (UTZINGER et al., 2009; ADENOWO et al., 2015).

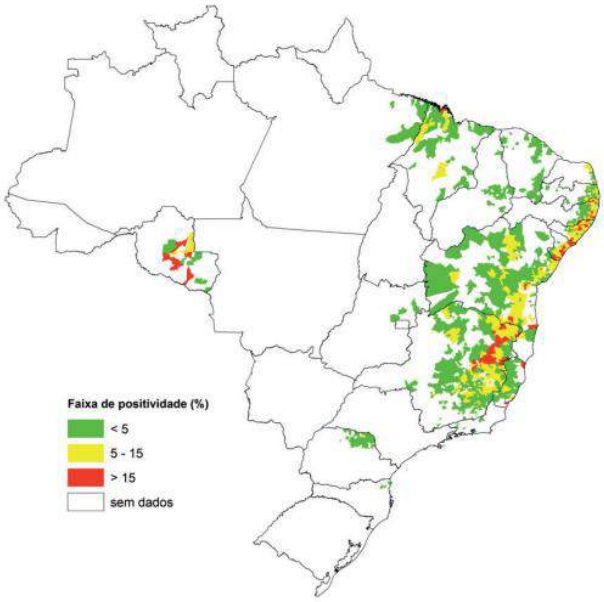
A descoberta da presença de focos de transmissão de esquistossomose na Europa é recente. Em março de 2014, casos de esquistossomose urogenital humana, transmitidos por meio de caramujos *Bulinus* de água doce, foram diagnosticados simultaneamente em hospitais franceses e alemães. Esses pacientes nunca haviam visitado um país endêmico de *S. haematobium*, ou seja, África e Península Arábica, mas todos os indivíduos infectados estiveram em contato com o rio Cavu (sul da Córsega). Após uma enorme campanha de diagnóstico em abril de 2015, mais de 100 casos adicionais que haviam contraído a doença durante o verão de 2013 foram relatados na França (BOISSIER, et al., 2015; KINCAID-SMITH, 2017).

No Brasil, encontram-se 96% dos casos de esquistossomose de toda América Latina e Caribe (MARTINS-MELO et al., 2015). Outros focos de transmissão no continente americano são encontrados na Colômbia, Venezuela, Porto Rico, República Dominicana, Santa Lúcia, Guadalupe, Martinica, St. Kitts, Suriname, Montserrat, Haiti e San Martin (SOUZA et al., 2007; CARDIM, 2010; NOYA et al., 2015).

No Brasil, a esquistossomose está distribuída em terras contínuas e contíguas, em quase toda a costa litorânea desde o Rio Grande do Norte até o Sul do país, se interiorizando alcançando Minas Gerais, no Sudeste, acompanhando várias bacias hidrográficas importantes nessas regiões. Focos da doença podem ser observados nos Estados do Ceará, Pernambuco, Paraíba, Alagoas, Sergipe, Bahia, Piauí, Maranhão, Pará, Rondônia, Goiás, Distrito Federal,

Espírito Santo, São Paulo, Rio de Janeiro, Minas Gerais, Paraná, Santa Catarina e Rio Grande do Sul (Figura 2) (MINISTÉRIO DA SAÚDE, 2014).

Figura 2 - Distribuição da esquistossomose no Brasil.



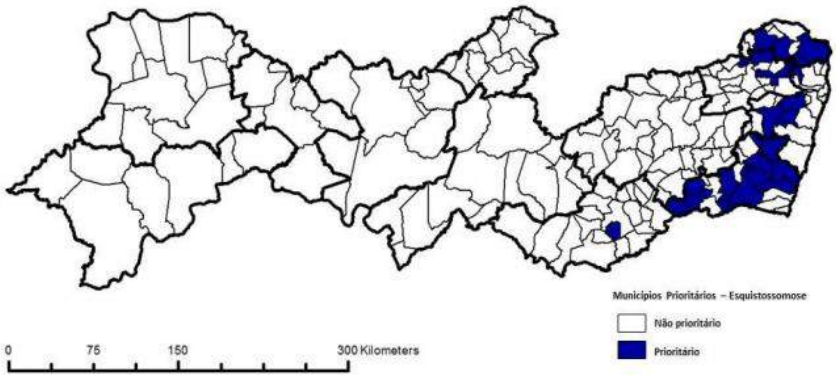
Fonte: MINISTÉRIO DA SAÚDE (2014).

Estima-se que cerca de 2,5 a 6 milhões de pessoas estejam infectadas no Brasil (destes, 5 a 10% podem desenvolver formas hepatoesplênicas graves e potencialmente fatais) e 25 milhões que vivem em áreas endêmicas correm risco de infecção. Entre 1990 e 2010, registrou-se um número significativo de formas graves, com uma média de 1.567 internações anuais e 527 óbitos (MARTINS-MELO et al., 2014; MINISTÉRIO DA SAÚDE, 2012).

Pernambuco é considerado o estado brasileiro com maior grau de endemicidade, concentrando cerca de 1/3 dos óbitos e apresentando uma série histórica (1979- 2010) de taxa de mortalidade de cerca de cinco vezes maior que a taxa nacional. De acordo com o SISPCE (Sistema de Informação do Programa de Controle da Esquistossomose), em 2014 foram realizados 256.660 exames parasitológicos de fezes em 117 municípios. A positividade de *Schistosoma mansoni* variou de 0,1% a 22,0% nos municípios endêmicos. Em 2017, foram definidas como prioritárias 56 localidades distribuídas em 26 municípios que apresentaram Índice de Positividade (IP) ≥10% em pelo menos dois anos no período de 2010-2014 (Figura 3) (SECRETARIA ESTADUAL DE SAÚDE DE PERNAMBUCO, 2017). Dos 185 municípios pernambucanos, 103 são endêmicos para esquistossomose e destes, 46,1%

pertencem à mesorregião do Agreste de Pernambuco, 41,2% estão na mesorregião da Zona da Mata Pernambucana e 12,7% na mesorregião metropolitana do Recife (MINISTÉRIO DA SAÚDE, 2014).

Figura 3 - Distribuição geográfica dos municípios prioritários para esquistossomose.



Fonte: SECRETARIA ESTADUAL DE SAÚDE DE PERNAMBUCO (2017)

Desde a década de 1990, pesquisas sobre a prevalência da esquistossomose são realizadas na Região Metropolitana do Recife. Estas pesquisas revelam o aparecimento sistemático de novos focos de transmissão ativos da esquistossomose em áreas periurbanas e litorâneas, indicando a expansão e descontrole da doença no estado de Pernambuco (OLIVEIRA et al., 2018).

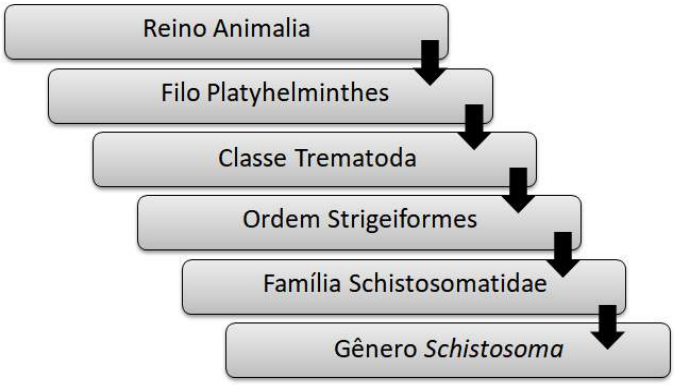
2.3*Schistosoma mansoni*, ASPECTOS GERAIS

A família Schistosomatidae engloba 13 gêneros de trematódeos que, em sua fase adulta, vivem no sistema venoso de crocodilos, aves e mamíferos. Entre as mais de 18.000 espécies de trematodas digenéicos (Filo Platyhelminthes, Classe Trematoda, Subclasse Digenea), os esquistossomos, juntamente com as famílias Spirorchiidae (em tartarugas) e Sanguinicolidae (em peixes) se diferenciam das demais, pois possuem dois hospedeiros (um caracol hospedeiro intermediário e um hospedeiro definitivo vertebrado), realizam a penetração direta na pele desses hospedeiros (em suas formas cercarianas), e eliminam ovos operculados, com espinho lateral ou terminal, e embrionados (miracídio). Estas famílias não possuem o estágio de metacercária e o terceiro hospedeiro, como é o caso dos outros trematódeos digeneicos. Os esquistossomos também têm sexos separados (são dioicos), em

nítido contraste com os Spirorchiidae, Sanguinicolidae e quase todos os Digenea restantes, que são monoicos (LOKER; MKOJI, 2005; SILVA; NEVES; GOMES, 2008).

Englobado na família Schistosomatidae, o gênero *Schistosoma* (Figura 4) apresenta diversas espécies descritas. No ano de 1994, a OMS iniciou o "*Schistosoma* Genome Network", o qual levou à descoberta do genoma do parasito. Os primeiros dados, publicados em 2009, evidenciaram 7 pares de cromossomos autossômicos e um par de cromossomos sexuais (ZW fêmea e ZZ macho) (BERRIMAN et al., 2009). Posteriormente, o sequenciamento do DNA mitocondrial fundamentou a classificação das 21 espécies de *Schistosoma* encontradas (YOUNG et al., 2012)

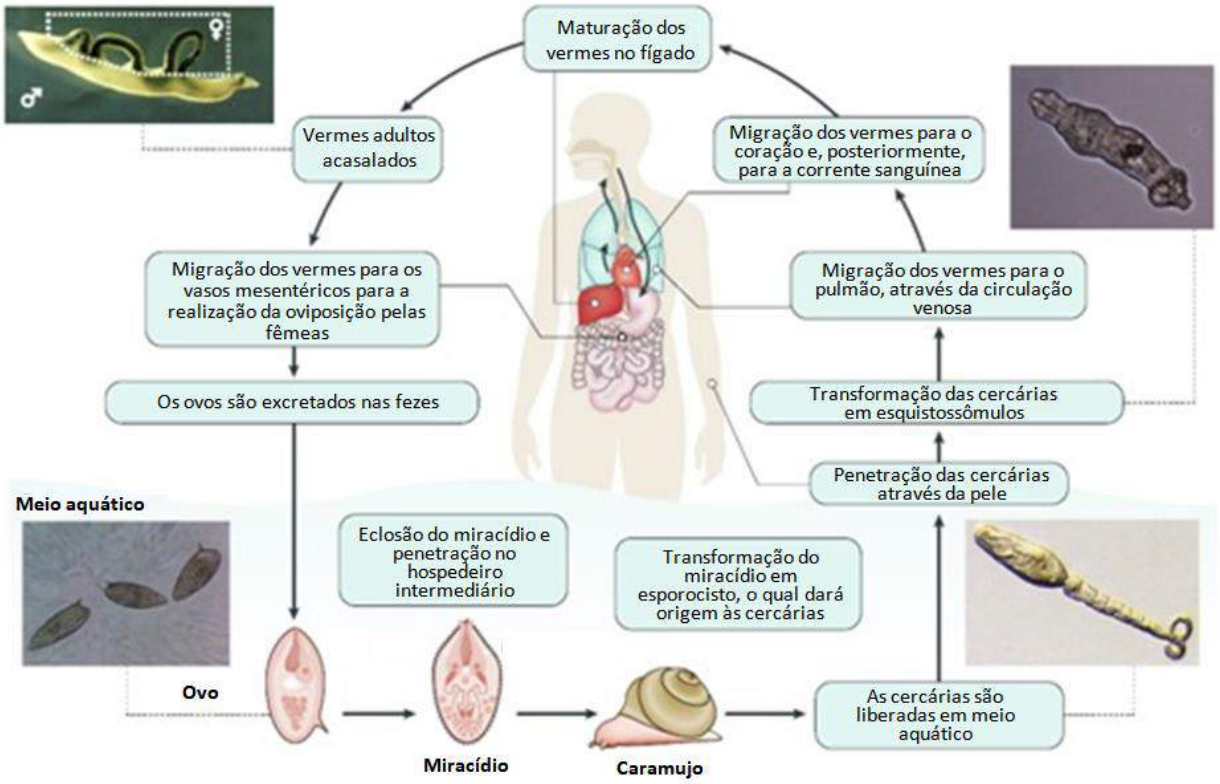
Figura 4 – Classificação taxonômica do gênero *Schistosoma*



2.3.1 Ciclo biológico de *Schistosoma mansoni*

O *S. mansoni* apresenta um complexo ciclo biológico (Figura 5), demonstrando uma excelente capacidade adaptativa ao meio ambiente e aos seus hospedeiros intermediários e definitivos (NEVES, 2005). O seu ciclo de vida é heteroxênico, sendo composto por duas fases: uma fase sexuada e outra fase assexuada. A fase assexuada tem início quando fezes de indivíduos infectados contendo ovos são liberados no meio ambiente em coleção hídrica. Dentro das condições ideais de temperatura, luminosidade e oxigenação, o miracídio, fase larval ciliada e infectante para o hospedeiro intermediário, eclode dos ovos. Guiados por estímulos luminosos e químicos, esses miracídios nadam ativamente em busca de caramujos do gênero *Biomphalaria*, penetrando em seus tegumentos em um processo que dura, aproximadamente, 15 minutos (COLLEY et al., 2014).

Figura 5 – Ciclo biológico de *Schistosoma mansoni*



Fonte: MCMANUS et al. (2018) (com modificações).

No molusco, o miracídio, após sofrer as tranformações morfológicas e fisiológicas iniciais, se transforma em esporocisto primário, que por poliembrionia origina esporocistos secundários, os quais migram para as glândulas digestivas e ovoteste do molusco. Cada esporocisto dará origem a uma numerosa quantidade de cercárias por reprodução assexuada. Um único miracídio tem potencial de originar mais de 100.000 cercárias . Todo esse processo evolutivo no hospedeiro intermediário dura cerca de quatro semanas (SOUZA et al., 2011). As cercárias saem do hospedeiro intermediário através da formação de vesículas no tegumento do molusco. Tal processo ocorre nos períodos mais quentes e luminosos do dia, sendo sua sobrevida limitada em cerca de dois dias e seu período de maior infectividade ocorrendo nas primeiras 8 horas após sua liberação em meio aquático (NEVES, 2005; SOUZA et al., 2011).

A fase sexuada se inicia com a penetração de cercárias (segunda fase larval do parasito e infectante para o hospedeiro definitivo) através da pele e mucosas do hospedeiro, o qual

entrou em contato com coleções aquáticas contaminadas. Durante o processo de penetração, as cercárias se fixam na pele ou mucosas do hospedeiro com o auxílio de suas duas ventosas (oral e ventral) e da liberação de uma substância mucoprotéica secretada por suas glândulas acetabulares. Ao atingir os capilares subcutâneos, o parasito perde a cauda bífida, se transformando em esquistossômulo, sendo levado pela corrente sanguínea ao lado direito do coração e, consequentemente, alcançando os capilares pulmonares (BARSOUM; ESMAT; EL-BAZ, 2013).

Após atingir os pulmões, os esquistossômulos migram para o sistema porta, podendo seguir este percurso por duas vias: (1) na via sanguínea, comumente mais aceita e de maior importância, os esquistossômulos saem das arteríolas pulmonares e dos capilares alveolares, ganhando a pequena circulação e chegando ao lado esquerdo do coração. Ganham a grande circulação através do fluxo aórtico e chegam até o sistema porta intra-hepático e ali se fixam. Já pela (2) via transtissular, os esquistossômulos saem dos alvéolos pulmonares através da penetração do parênquima pulmonar, pleura e diafragma, alcançando a cavidade peritoneal e perfurando a cápsula e parênquima hepático, chegando ao sistema porta intra-hepático (NEVES, 2005).

No sistema porta intra-hepático, intensificam o processo de nutrição e se desenvolvem, diferenciando-se morfologicamente em machos e fêmeas após 25-28 dias após a infecção. Após o amadurecimento, os vermes, acasalados, migram até às vênulas terminais do plexo mesentérico inferior, onde a fêmea realizará o processo de oviposição. O tempo de vida de *S. mansoni* é de cerca de 5 anos, entretanto existem relatos de que casais de vermes já atingiram os 30 anos de vida produzindo ovos ativamente (GRYSSELS at al., 2006). Os primeiros ovos podem ser vistos nas fezes de indivíduos infectados após 45 dias da penetração cercariana (NEVES, 2005; SOUZA et al., 2011). Se os ovos contidos nas fezes encontrarem novamente o ambiente aquático, o ciclo se reinicia.

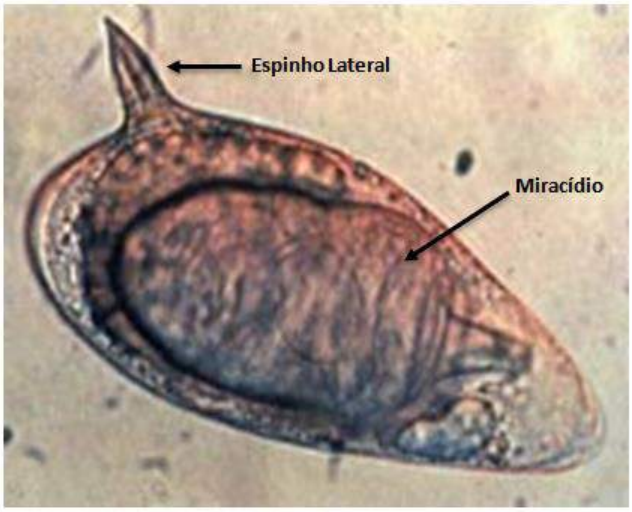
2.3.2 Morfologia das fases evolutivas de *Schistosoma mansoni*

A morfologia do gênero *Schistosoma* varia de acordo com a fase evolutiva do parasito: ovo, miracídio, esporocisto, cercária, esquistossômulo e vermes jovens e adultos. Na espécie *S. mansoni*, a morfologia é descrita da seguinte forma:

- Ovo

Possui formato oval e mede cerca de 150 µm de comprimento por 60 µm de largura, não apresenta opérculo e, em sua parte mais larga, possui um espinho lateral. No momento da postura, os ovos contêm um embrião em formação, entretanto apenas passados 6-7 dias, o miracídio (larva ciliada) no ovo se torna maduro. A presença de um miracídio devidamente formado em seu interior lhe caracteriza como ovo maduro, sendo a forma comumente encontrada nas fezes (Figura 6). Ovos maduros possuem tempo de vida médio de 20 dias nos tecidos do hospedeiro, morrendo o miracídio caso não ocorra sua expulsão nas fezes (NEVES, 2005; MINISTÉRIO DA SAÚDE, 2014).

Figura 6 – Ovo de *Schistosoma mansoni*

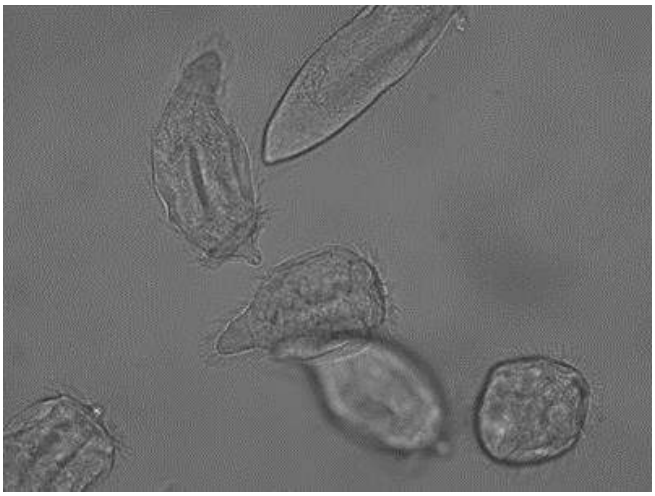


FONTE: http://lineu.icb.usp.br (com modificações). Acesso em 05.04.2018.

- Miracídio

Possui forma cilindrocônica (Figura 7), medindo cerca de 160 – 180 µm de comprimento por 60 µm de largura. A sua superfície tem 21 placas epidérmicas ciliadas, sem núcleo, e agrupadas em quatro fileiras, cujos cílios aumentam de tamanho da região anterior para a posterior. Em sua região anterior, o miracídio consiste de uma papila apical em formato cônico (terebratorium), representado por pregas anastomosadas do tegumento, e contendo glândulas adesivas e de penetração, juntamente com diversos receptores sensoriais (SILVA; NEVES; GOMES, 2008).

Figura 7 – Miracídio de *Schistosoma mansoni*



FONTE: http://www.fiocruz.br. Acesso em 05.04.2018.

Seu aparelho excretor é composto por células solenócitos (células flama), dispostas em em dois pares e unidas por pequenos canais que são drenados para uma ampola excretora, que culmina no poro excretor (NEVES, 2005).

Possui sistema nervoso primitivo, formado por uma junção de fibras conectadas às células nervosas por um grande gânglio cefálico e composto por numerosas fibras nervosas com corpos celulares periféricos. A contratilidade e motilidade da larva são comandadas por este sistema (SILVA; NEVES; GOMES, 2008).

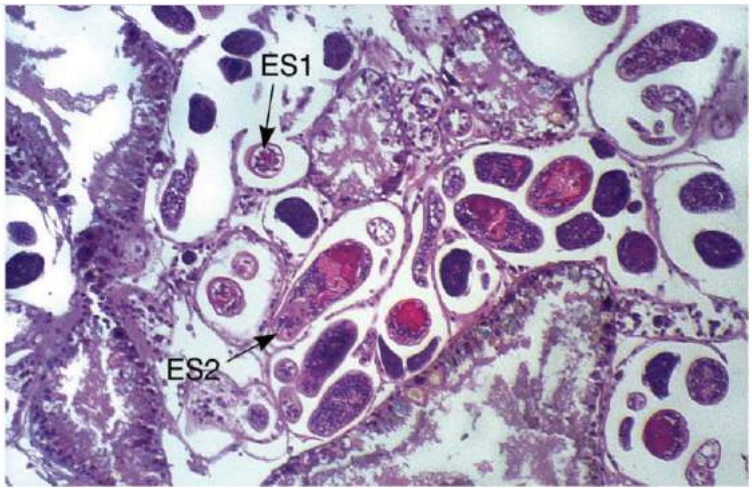
- Esporocisto

A modificação de ambiente do miracídio, após a penetração em um molusco, exige uma adaptação às novas condições fisiológicas, como temperatura, pH, oxigenação, osmolaridade, etc. As células germinativas (de 50 a 100 células) iniciam um intenso processo de multiplicação chamado de poliembrionia. Assim sendo, os miracídios sofrem rápidas transformações estruturais na proximidade do seu ponto de penetração para a formação do esporocisto primário. Permanecendo no mesmo local, crescem e se transformam em um saco alongado, e após 2 horas da penetração, perdem seu revestimento epitelial ciliado e demais estruturas externas. No segundo dia, uma nova membrana surge em sua superfície, na qual aparecem inúmeras dobras semelhantes à microvilosidades cobertas por uma camada eletrodensa que realiza trocas metabólicas com a hemolinfa do molusco. O novo tegumento

sincicial contém mitocôndrias, ribossomos, retículo endoplasmático, glicogênio e lipídeos (SILVA; NEVES; GOMES, 2008).

Na segunda semana de infecção, uma série de ramificações tubulares se formam no interior do esporocisto, preenchendo todos os espaços intercelulares do tecido conjuntivo. No interior dessas ramificações, as células germinativas permanecem em intensa multiplicação. Em condições climáticas ideais, a formação do esporocisto secundário ocorre a partir do 14º dia após a penetração do miracídio no hospedeiro intermediário. O desenvolvimento do esporocisto secundário se inicia com um aglomerado de células germinativas nas paredes do esporocisto primário, podendo ser observada uma vacuolização proeminente na região central da larva. Esses aglomerados se reorganizam, dando origem a septos, sendo o esporocisto primário dividido em 150 a 200 camadas, e podendo cada septo ou camada já ser chamado de esporocisto secundário (NEVES, 2005; SILVA; NEVES; GOMES, 2008) (Figura 8).

Figura 8 – Esporocistos de *Schistosoma mansoni*. ES1 – Esporocisto primário. ES2 – Esporocisto secundário.



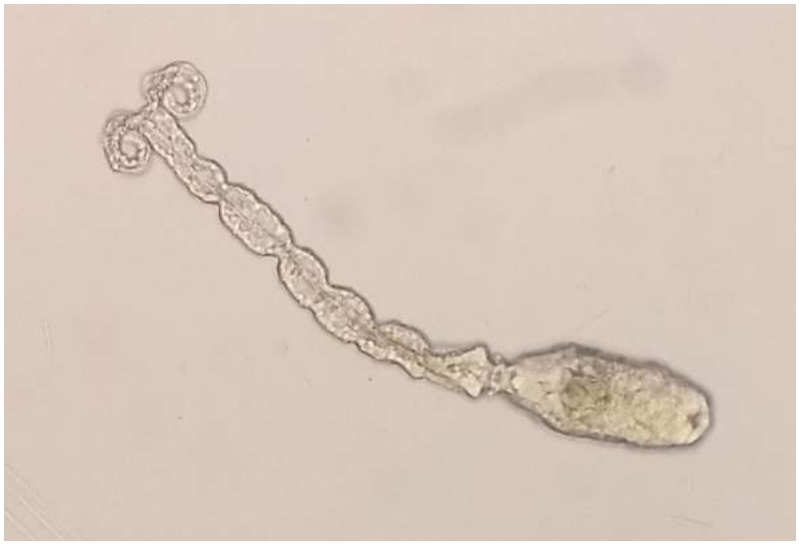
Fonte: SILVA; NEVES; GOMES (2008)

- Cercária

As cercárias (Figura 9) apresentam, aproximadamente, 500 µm de comprimento (cauda bifurcada medindo 230 por 50 µm e corpo cercariano medindo 190 por 70 µm). Exibem duas ventosas: uma oral e uma ventral. Se apresentam cobertas de pequenos espinhos, onde o número e saliência das projeções desses espinhos se mostram menos proeminentes no corpo que na cauda, demonstrando diferença na composição do tegumento. A presença de um "colar" de receptores sensoriais composto por cílios e medindo, aproximadamente, 2,18 µm

no corpo cercariano é observado, assim como 8 pares de glândulas sensoriais que podem ser retraídas ou projetadas através da ventosa oral. A ventosa ventral (ou acetábulo) é a maior e possui musculatura mais desenvolvida, devido a sua função de fixação na pele do hospedeiro no processo depenetração. Possuem sistema excretor formado por quatro pares de células flama. Como a cauda é uma estrutura que se perderá rapidamente no processo de penetração, não possui estrutura complexa nem órgão definidos, servindo apenas para a movimentação da larva em meio aquático (PEREIRA et al., 2013; NEVES, 2005).

Figura 9 – Cercária de *Schistosoma mansoni*

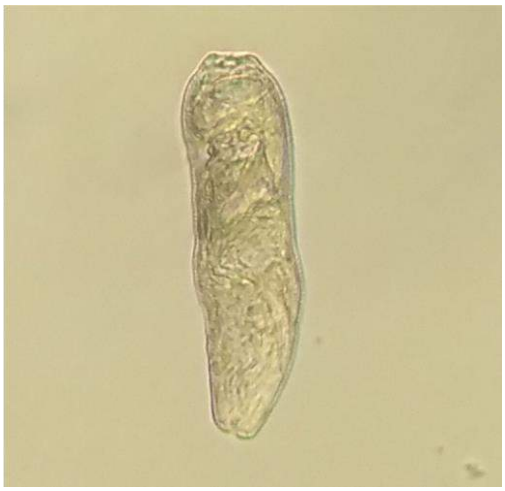


Fonte: AUTOR (2019)

- Esquistossômulo

Após penetrar no hospedeiro definitivo, a cercária perde a cauda dando início a uma série de transformações fisiológicas e ultraestruturais para a adaptação do parasito ao ambiente interno do hospedeiro. Algumas dessas mudanças é a perda do glicocálix, respiração anaeróbica, conversão da membrana tegumentar trilaminada para heptalaminada, perda da tolerância à agua e a evacuação de glândulas secretórias. Todas essas mudanças resultam no esquistossômulo (Figura 10) de pele após 2-3 horas da penetração. Após 3 horas, a maior parte da membrana externa é heptalaminada e o tegumento apresenta grandes vacúolos, corpos alongados e pequenos corpos membranosos. Após 24 horas, a principal modificação é a formação de pregas e cavidades na superfície (SILVA; NEVES; GOMES, 2008; TEKWU et al., 2016)

Figura 10 – Esquistossômulo de *Schistosoma mansoni*



Fonte: AUTOR (2019)

Após migrarem para os pulmões, cerca de 6 dias depois da penetração, os esquistossômulos se tornam maiores, com cerca de 400 µm de comprimento e mais estreitos do que os encontrados na pele, com as extremidades (anterior e posterior) recobertas de espinhos. A superfície do tegumento se torna pregueada e escavada e, após 14 dias da penetração, apresentam ceco preenchido por pigmento, resultante da digestão de hemoglobina. A área de superfície do tegumento do esquistossômulo pulmonar é cerca de quatro vezes maior que a área do esquistossômulo de pele de sete dias (SILVA; NEVES; GOMES, 2008).

- Vermes jovens

Após chegarem ao fígado, as transformações seguintes dão início à organogênese e formação dos vermes jovens (Figura 11), cerca de 21 dias após a penetração no hospedeiro definitivo. Os machos imaturos apresentam dois pequenos lobos testiculares e o canal ginecóforo parcialmente desenvolvido. Já nas fêmeas, o útero ainda é rudimentar e o tegumento é composto por largas estrias profundas e bem organizadas. Após quatro semanas a gametogênese ocorre. Machos apresentam oito lobos testiculares, com espermatozóides visíveis nos dois ou três lobos anteriores. A superfície do corpo se torna mais lisa, surgem organelas sensoriais e na sua região dorsal surgem protuberâncias denominadas de tubérculos. Nas fêmeas há um pequeno ovário e o início do desenvolvimento do oótipo. Na quinta semana, os machos não apresentam mais modificações importantes em seus sistemas

reprodutores. O canal ginecóforo se forma quando as extremidades do corpo dobram entre si, da esquerda para a direita ou vice-versa. A região dorsal já se encontra recoberta de inúmeros tubérculos, separados por uma superfície dotada de estrias rasas. Nas fêmeas a proteína da casca do ovo aparece e os folículos ovarianos se desenvolvem ao longo do corpo, posteriores ao ovário (SILVA; NEVES; GOMES, 2008).

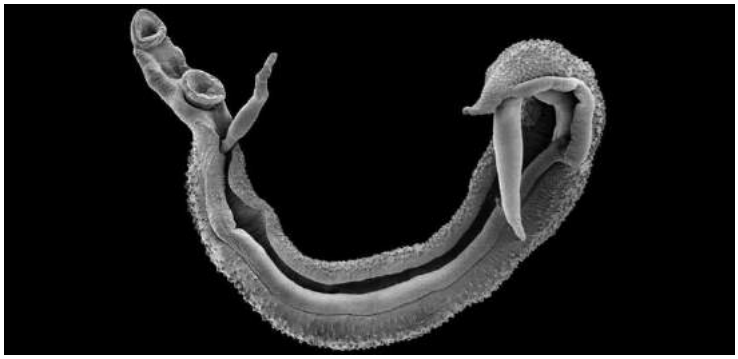
Figura 11 – Vermes *Schistosoma* mansoni jovens (21 dias)

Fonte: AUTOR (2019)

- Vermes Adultos

Os vermes machos possuem, aproximadamente, 1 cm de comprimento, coloração esbranquiçada, tendo seu tegumento recoberto por inúmeros tubérculos e corpo dividido em duas porções: anterior, região onde são encontradas as ventosas oral e ventral (acetábulo), e posterior, região que se inicia após a ventosa ventral e dá início ao canal ginecóforo. Após a ventosa oral, pode-se observar o esôfago, que se bifurca na região do acetábulo, fundindo-se e formando um único ceco, que culmina na extremidade posterior. Atrás do acetábulo se encontram de sete a nove massas testiculares que se abrem diretamente no canal ginecóforo (dobras das laterais do corpo no sentido longitudinal para albergar a fêmea). Não possuem órgão copulador, assim sendo, quando acasalado com a fêmea (Figura 12), os espermatozóides passam pelos canais deferentes, se abrindo no poro genital dentro do canal ginecóforo, alcançando a fêmea e fecundando-a (NEVES, 2005).

Figura 12 – Casal de *Schistosoma mansoni* adultos



Fonte: http://www.cdipd.org. Acesso em 05.04.2018.

Vermes fêmeas possuem uma coloração mais escurecida que a do macho, medindo cerca de 1,5 cm de comprimento, é cilindrica com extremidades afiladas, possuindo tegumento liso com discretas estrias paralelas. Na região anterior se encontram as ventosas oral e ventral. No início da porção posterior observa-se em sequência a vulva, útero e o ovário. Toda porção posterior é preenchida pelas glândulas vitelogênicas (ou vitelinas) e o ceco (NEVES, 2005; MINISTÉRIO DA SAÚDE, 2014).

2.5 ASPECTOS CLÍNICOS E PATOLÓGICOS DA ESQUISTOSSOMOSE MANSÔNICA

O quadro e a evolução do processo patológico na esquistossomose varia com uma série de circunstâncias: linhagem do parasito, carga infectante, condições fisiológicas das cercárias no momento da infecção, características do hospedeiro (idade, aspectos nutricionais, hábitos e condições de vida), ocorrência ou não de infecções anteriores e grau de imunidade do hospedeiro (REY, 2001). Os variados aspectos patogênicos e patológicos observados na esquistossomose também vão depender da interação parasito-hospedeiro nas 4 fases evolutivas do verme que entram em contato com o hospedeiro humano: cercárias, esquistossômulo, vermes adultos e ovos (MORAES, 2011).

Logo após a infecção, o indivíduo infectado pode apresentar dermatite cercariana, de duração geralmente transitória (24 a 72 horas), caracterizada por micropápulas eritematosas e pruriginosas, com intensidade e duração geralmente pequenas (LAMBERTUCCI; SILVA; VOIETA, 2005; AIRES, 2013).

Posteriormente, há um período de incubação no qual ocorre o desenvolvimento das fases imaturas do verme (esquistossômulos) e sua posterior eliminação para a corrente sanguínea e migração para os pulmões. Nessa fase, pode ocorrer linfadenia generalizada e

aumento volumétrico do baço. Parte dos pacientes passam por esse período de forma assintomática, entretanto outros podem apresentar mal-estar, com ou sem febre, problemas pulmonares (tosse), dores musculares, desconforto abdominal e um quadro de hepatite aguda causada pela formação de produtos oriundos da destruição dos esquistossômulos pelo sistema imune (NEVES, 2005).

Por volta do 15º ao 25º dia após a infecção, quando os vermes se encontram em sua fase juvenil, pode ocorrer febre, eosinofilia, linfadenopatia, esplenomegalia e urticária. O fígado pode apresentar um processo inicial de hepatite com volume ligeiramente aumentado. A esplenomegalia possui características de uma esplenite infecciosa aguda, com infiltração eosinofílica abundante. As alterações intestinais também começam a surgir antes do período postural, compreendendo numerosas ulcerações necróticas hemorrágicas da mucosa, com pequenas úlceras disseminadas por todo o intestino. Pode ser observada hipertrofia ganglionar generalizada, aumento das células imunocompetentes, e das gamaglobulinas, com inversão da relação albumina/globulina. Quando a doença progride, e se inicia o período pós-postural, há a disseminação de ovos em diversos órgãos associados à reações granulomatosas (REY, 2001).

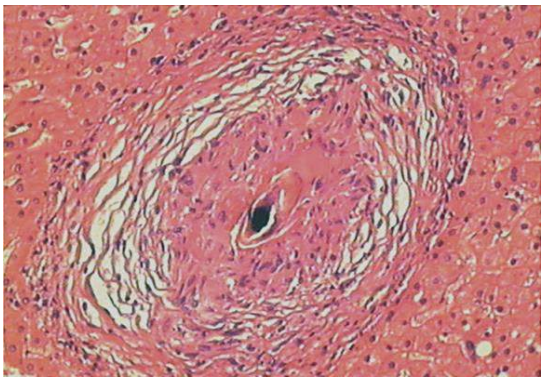
Na fase aguda da doença, em torno de 50 dias após a infecção, ocorre uma disseminação volumosa de ovos, com destaque nas paredes intestinais, podendo ser observadas áreas de necrose, ocasionado uma enterocolite aguda, e surgimento de granulomas hepáticos, caracterizando a forma toxêmica, que pode apresentar sinais clínicos de febre, acompanhada de sudorese, calafrios, emagrecimento, fenômenos alérgicos, diarreia, disenteria, cólicas, tenesmo, hepatoesplenomegalia discreta, linfadenia, leucocitose com eosinofilia, aumento das globulinas e alterações discretas das transaminases. Em casos mais graves, pode até haver casos de óbito na fase toxêmica, ou então, como é observado na maioria dos casos, ocorre a evolução da doença para a fase crônica, cuja evolução é lenta (NEVES, 2005).

Depois da fase aguda, os sintomas regridem, permanecendo a maioria dos pacientes assintomáticos. As manifestações clínicas ocorrem, em sua maioria, mais tardiamente, devido às repetidas exposições do paciente à reinfecção nos focos endêmicos, com o consequente aumento da carga parasitária. Nessa fase, as manifestações clínicas da doença são predominantemente intestinais e hepatointestinais (REY, 2001). Na fase crônica da esquistossomose, em muitos casos, o paciente apresenta diarreia mucossanguinolenta, dor abdominal e tenesmo. Nos casos crônicos mais graves, pode ser observada a fibrose da alça retossigmóide, levando à diminuição do peristaltismo e constipação constante. A diarreia

mucossanguinolenta é resultado da passagem simultânea de um grande número de ovos para a luz do intestino, originando pequenas (mas numerosas) hemorragias e edema (NEVES, 2005).

O elemento anatomopatológico básico do processo esquistossomótico crônico, o granuloma (Figura 13), que surge em torno dos ovos do parasita em função de uma reação inflamatória, demonstra a importância do ovo como agente patogênico (REY, 2001). As lesões hepatoesplênicas são oriundas, principalmente, da formação desses granulomas, produtos da hipersensibilidade do hospedeiro aos antígenos secretados pelo miracídio, presente no interior do ovo, e denominados genericamente de SEA (*Soluble egg antigens* – antígenos solúveis do ovo) (AIRES, 2013).

Figura 13 – Granuloma periovular hepático em paciente infectado com *Schistosoma mansoni*



Fonte: ANDRADE, 2008

Muitos dos granulomas formados em torno dos ovos arrastados pela corrente sanguínea e que ficaram retidos nos capilares dos espaços porta do fígado, acabam por dar início ao desenvolvimento uma neoformação conjuntivo-vascular, onde se observa uma retração da cápsula hepática por fibrosamento dos espaços porta, em torno das ramificações intra-hepáticas da veia porta. Esta disposição sistematizada do tecido reacional é denominada de fibrose periportal (fibrose de Symmers), sendo considerada, também, um importante elemento anatomopatológico do curso da fase crônica da esquistossomose (REY, 2001; NEVES, 2005).

Uma das mais importantes consequências da fibrose hepática, devido uma maior dificuldade do fluxo venoso nesse local, é a hipertensão na veia porta e em todo território drenado por ela, acarretando congestão e edema na parede do estômago e intestinos, congestão e aumento do volume esplênico (esplenomegalia), bem como alterações na circulação e atividade fisiológica de outros órgãos da cavidade abdominal. Quando a

dificuldade circulatória aumenta, o sangue abre passagem através das anastomoses entre os sistemas porta e cava inferior e entre os sistemas porta e cava superior. Essa formação circulatória colateral leva à formação de veias varicosas de grande calibre na parede esofágica, sujeitas à ruptura e hemorragias grave que podem levar o paciente a óbito (REY, 2001; AIRES, 2013).

2.6 HOSPEDEIROS INTERMEDIÁRIOS DE *Schistosoma mansoni*

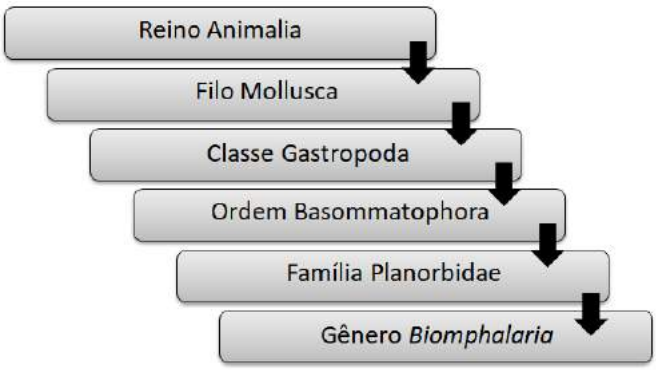
2.6.1 Aspectos biológicos do gênero *Biomphalaria*

Quatro gêneros de moluscos podem albergar parasitos *Schistosoma* de importância médica e epidemiológica, servindo como hospedeiros intermediários de seu ciclo de vida, são eles: os moluscos do gênero *Biomphalaria* . (*S. mansoni*), *Bulinus* (*S. haematobium* e *S. intercalatum*), *Oncomelania* (*S. japonicum*), e a espécie *Neotricula aperta* (*S. mekongi*) (ROZENDAAL, 1997; ATTWOOD; UPATHAM, 2012).

No Brasil, três espécies do gênero *Biomphalaria* são encontradas naturalmente infectadas por *S. mansoni*: *Biomphalaria glabrata* (Say, 1818), *Biomphalaria tenagophila* (D'orbigny, 1835) e *Biomphalaria straminea* (Dunker, 1848). Infecções experimentais foram documentadas nas espécies *Biomphalaria peregrina* (D'orbigny, 1835), *Biomphalaria amazonica* (Paraense, 1966) e *Biomphalaria cousini* (Paraense, 1966) (MINISTÉRIO DA SAÚDE, 2014).

Segundo Rey (2001), a classificação taxonômica do gênero *Biomphalaria* é descrita de acordo com a Figura 14.

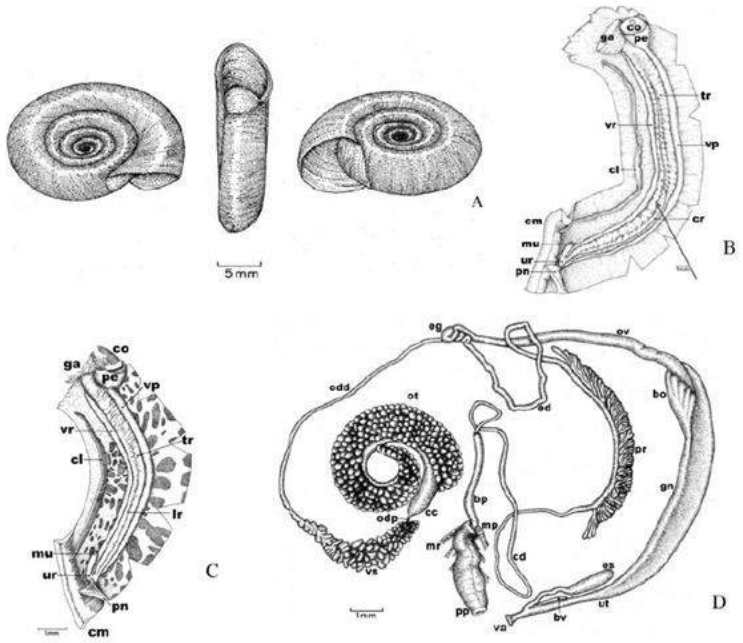
Figura 14 – Classificação Taxonômica do Gênero *Biomphalaria*



Fonte: REY (2001)

Apesar de não possuírem uma constituição fisiológica e morfológica complexa (Figura 15), a realização das atividades vitais (digestão, respiração, excreção, circulação e reprodução) pelas espécies do gênero é bastante eficiente, garantindo a sobrevivência e proliferação das espécies sob diversos panoramas ambientais, condições climáticas ou ecológicas. O sistema digestivo é completo, com boca e ânus. Possuem estômago e intestino bem desenvolvidos, e uma glândula digestiva, também denominada de hepatopâncreas. A obtenção do alimento é realizada através de raspagens com a rádula, estrutura provida de dentes quitinosos em linhas transversais e longitudinais (TELES; CARVALHO, 2008). A digestão extracelular ocorre no estômago, devido à ação de enzimas provenientes das bolsas esofágicas e do ceco digestivo. O intestino estende-se da extremidade anterior do estômago através da massa visceral, para se abrir via ânus, do lado direito da cavidade do manto. A porção terminal do sistema digestivo é o reto, que tem por função a formação, compactação e armazenamento das fezes (BARNES; RUPPERT, 1996; MOORE, 2003).

Figura 15 - Desenho esquemático da concha e da morfologia interna de *Biomphalaria glabrata*



A) Desenho da concha de *B. glabrata*: vista do lado direito, vista frontal e vista do lado esquerdo, respectivamente; B) Manto de um exemplar adulto, onde se vê a crista renal; C) Manto de um exemplar jovem com linha renal pigmentada; D) Sistema reprodutor: canal coletor do ovoteste (cc), encruzilhada genital (eg), ovispermiduto proximal (odp), ovispermiduto distal (odd), ovoteste (ot) e vesícula seminal (vs); estruturas masculinas: bainha do pênis (bp), canal deferente (cd), espermiduto (ed), músculos do complexo peniano [retrator (mr) e protrator (mp)], prepúcio (pp) e próstata (pr); estruturas femininas: bolsa do oviduto (bo), bolsa vaginal (bv), espermateca (es), glândula nidamental (gn), oviduto (ov), vagina (va) e útero (ut); coração (co), pericárdio (pe), glândula do albúmen (ga), veia pulmonar (vp), veia renal (vr), tubo renal (tr), crista lateral (cl), crista renal (cr), linha renal pigmentada (lr), colar do manto (cm), ureter (ur), meato do ureter (mu) e pneumóstoma (pn).

Fonte: PARAENSE (1975) (com modificações)

A respiração é realizada através do saco pulmonar, principal órgão respiratório, e as pseudobrânquias, pregas tegumentares ricas em vasos. A respiração atmosférica é predominante, onde a hematose ocorre na rede vascular da parede pulmonar, onde o sangue flui através da veia pulmonar. A respiração aquática ocorre através das pseudobrânquias e do tegumento em contato com meio líquido (MINISTÉRIO DA SAÚDE, 2008).

O rim é uma estrutura alongada, com inúmeros septos e granulações internas, iniciando-se na cavidade pericárdica até a borda do manto, terminado no ureter, que se abre próximo ao ânus e pneumostômio. O rim é dotado de uma prega mucosa, saliente e pigmentada, denominada de crista renal (BARNES; RUPPERT, 1996; REY, 2001; NEVES, 2005).

O sistema nervoso é formado por 11 gânglios, sendo 5 deles dispostos em pares (bucais, cerebrais, pleurais, pedais e parietais) e um deles disposto isoladamente (visceral). Esses gânglios formam um "anel" em volta do esôfago (anel periesofagiano), logo atrás do saco bucal. Os gânglios estão acomodados em uma cápsula de tecido conjuntivo e ligados a receptores sensoriais através de neurônios aferentes ou sensoriais, e a músculos e glândulas por neurônios eferentes ou motores. Os órgãos sensoriais destes moluscos incluem olhos, receptores de contato (tentáculos), um par de órgãos do equilíbrio e orientação locomotora (otocistos), osfrádios (órgão quimiossensorial) e quimiorreceptores, modificados ou não, denominados de rinóforos (REY, 2001; CIMERMAM; CIMERMAM, 2001; BARNES; RUPPERT, 1996).

Os caramujos são hermafroditas, realizando, preferencialmente, a fecundação cruzada, que propicia maior variabilidade genética, e, em casos isolados, a autofecundação, a qual garante a formação de uma população a partir de um único indivíduo (MINISTÉRIO DA SAÚDE, 2008). Possuem gônada única em forma de cachos, denominada ovoteste, no qual os óvulos e espermatozóides são produzidos lado a lado, entretanto a produção simultânea de gametas não ocorre, sendo produzidos em alternância. O ovoteste possui diversos folículos, sendo cada um uma unidade de produção dos dois gametas (CANTINHA, 2008).

O sistema circulatório desses animais é aberto e composto por coração com duas cavidades: aurícula e ventrículo. Possuem hemolinfa rica em hemoglobina (representando cerca de 97% de todas as proteínas presentes na hemolinfa), água, cloreto de sódio e bicarbonatos (PESSÔA; MARTINS, 2011; REY, 2001; VERRENGIA GUERRERO et al., 1997). Os componentes celulares presentes na hemolinfa são denominados de hemócitos, principais células efetoras do sistema de defesa desses animais, as quais se movem livremente através dos tecidos e exercem atividades de fagocitose, encapsulação, liberação de substâncias

citotóxicas, transporte de cálcio no reparo tecidual e na produção de fatores coagulantes. Estas células lembram, em sua forma e função, os macrófagos dos animais vertebrados (ABDUL-SALAM; MICHELSON, 1980; AMEN et al., 1992; BAYNE; BUCKLEY; DEWAN, 1980; CAVALCANTI, 2011).

Caramujos pertencentes ao gênero *Biomphalaria* possuem concha em formato de disco de tamanhos variados a depender da espécie. A *B. glabrata* é a principal espécie hospedeira de *S. mansoni*, por ser a mais susceptível à infecção e a todas as linhagens geográficas de *S. mansoni*. É o maior molusco da família Planorbidae, com sua concha chegando a atingir até 40 mm de diâmetro , 11 mm de largura com seis a sete giros. A principal característica anatômica interna da espécie a presença de uma crista renal pigmentada localizada ao longo da superfície ventral do tubo renal em indivíduos adultos. A espécie *B. tenagophila* possui concha com até 35 mm de diâmetro, 11 mm de largura com sete a oito giros. Em relação à anatomia interna, são semelhantes aos *B. glabrata*, embora seu tubo renal seja um pouco mais alongado e sem a presença de crista renal. A *B. straminea* é a menor das três espécies, apresentando concha com até 16,5 mm de diâmetro, 6 mm de largura e cinco giros. A principal característica anatômica dessa espécie é a presença de enrugamento transversal nas paredes dorsal e esquerda da vagina, ausentes em *B. glabrata*. Também não possuem crista renal. (NEVES, 2005).

As espécies do gênero *Biomphalaria* comumente habitam coleções hídricas de água doce naturais, artificiais e temporárias, do tipo lêntico ou lótico, e de altitudes variadas, no mesmo nível ou acima do nível do mar. Apesar da grande variação de habitats, são mais facilmente observados próximo às margens de coleções hídricas de pequeno porte (TELES; CARVALHO, 2008). Optam por locais com boa disponibilidade de matéria orgânica, boa iluminação, temperatura média entre 20 a 26 ºC, se alimentando de folhas e algas (NEVES, 2005). A presença de vegetação vertical ou flutuante é de grande importância para a alimentação e abrigo dos animais, além de ser um dos substratos de preferência dos moluscos para depositar suas desovas em área submersa (MINISTÉRIO DA SAÚDE, 2014).

Os biótopos em que vivem esses moluscos, em sua maioria, tem pH em torno de 6,0 a 8,0. Em coleções aquáticas com pH inferior, os planorbídeos são incapazes de promover a deposição de cálcio para a formação da concha, o que impede sua colonização nesses habitats. Densidades populacionais elevadas desses animais geralmente são encontradas em águas mais duras, ricas em cálcio (AMARAL; THIENGO; PIERI, 2008).

Como resposta às modificações ambientais desfavoráveis, como secas e inundações, os moluscos do gênero *Biomphalaria* utilizam diversos mecanismos de sobrevivência: (1) a

anidrobiose (diminuição da atividade metabólica), que ocorre em processos lentos de dessecação da coleção hídrica, (2) o enterramento, onde o caramujo se enterra no solo de ambientes aquáticos em resposta à possíveis agressões do meio, como a aplicação de agentes moluscicidas, (3) a diapausa, onde o animal para de forma brusca o seu desenvolvimento, mesmo em condições favoráveis, e (4) a quiescência, parada do desenvolvimento do animal determinada diretamente por condições ambientais desfavoráveis, como o aumento (estivação) ou diminuição (hibernação) da temperatura. Mesmo em estado de anidrobiose, enterramento, diapausa ou quiescência, os caramujos podem carregar consigo as formas imaturas do *S. mansoni*, os quais retomam suas atividades quando as condições ambientais voltam à normalidade (MINISTÉRIO DA SAÚDE, 2014).

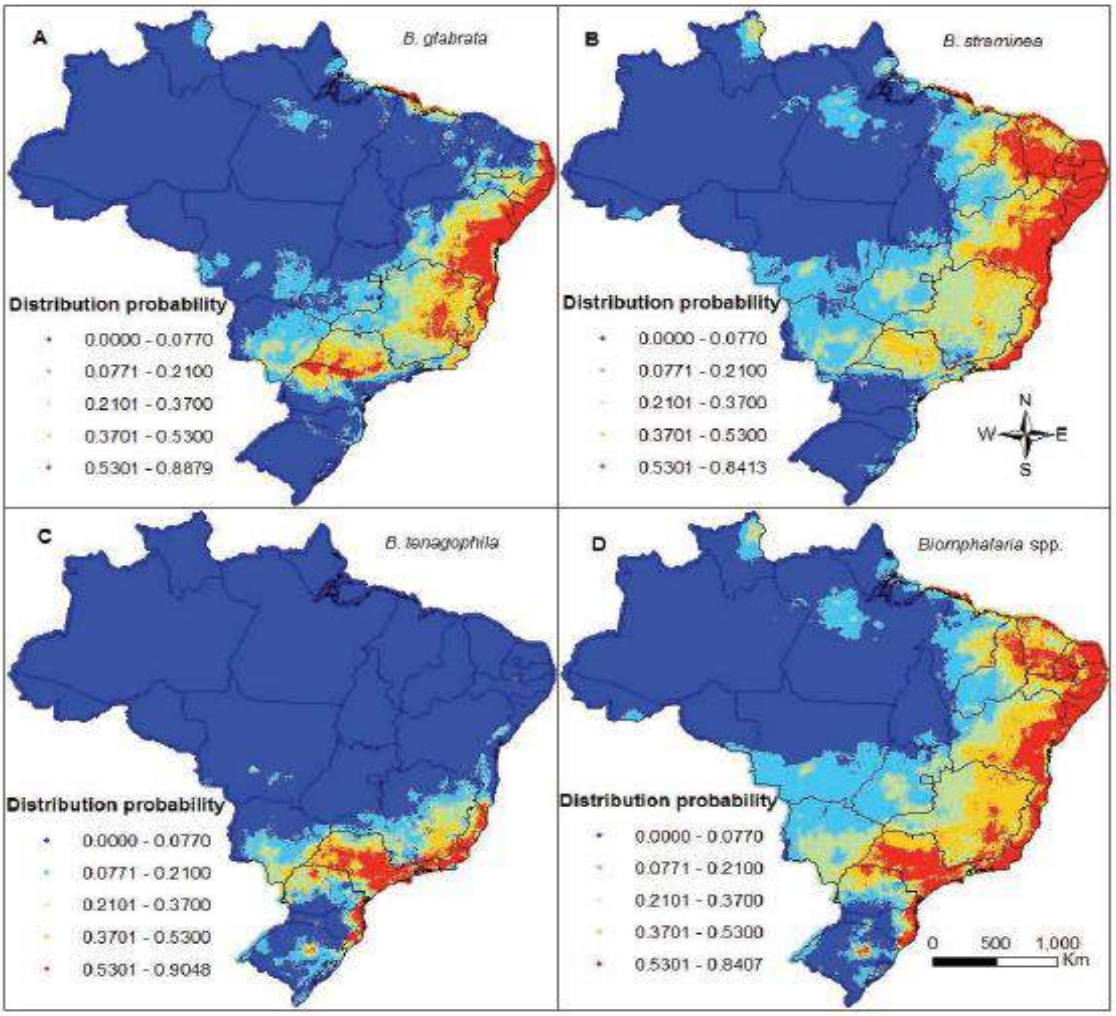
A sobrevida dos caramujos, geralmente, não ultrapassa um ano. Entretanto, a população consegue, com sucesso, ser mantida nos criadouros devido às eficientes estratégias reprodutivas dos animais. Em condições adversas, um único ou poucos indivíduos podem utilizar os mecanismos de autofecundação, dando início a uma nova população, podendo em apenas três meses alcançar cerca de 10 milhões de descendentes. São ovíparos, e intensificam a postura de desovas no período noturno, sendo os ovos depositados nos substratos em uma cápsula transparente. O número de ovos postos em cada desova varia de acordo com o animal em questão e fatores ambientais. O tempo entre a postura e a eclosão dos indivíduos ocorre em cerca de 6 a 10 dias (MINISTÉRIO DA SAÚDE, 2014). Possuem fases de desenvolvimento embrionário já bem elucidados e definidos, sendo os embriões classificados em: blástula (0 a 15 horas após a primeira clivagem), gástrula (24 a 39 horas após a primeira clivagem), trocófora (48 a 87 horas após a primeira clivagem) e véliger (96 a 111 horas após a primeira clivagem) (KAWANO; NAKANO; WATANABE, 2008).

2.6.2 Distribuição geográfica do gênero *Biomphalaria*

A espécie *B. glabrata* é considerada a mais importante hospedeira intermediária dos *S. mansoni* na América do Sul, devido ao grande percentual de moluscos infectados, bem como apresentar sua distribuição espacial sempre relacionada com áreas endêmicas para a esquistossomose, tendo sido encontrada em ambientes naturais com taxas de positividade de até 80%. Foi identificada em 16 estados brasileiros (Figura 16 - A): Alagoas, Bahia, Espírito Santo, Goiás, Maranhão, Minas Gerais, Pará, Paraíba, Paraná, Pernambuco, Piauí, Rio de Janeiro, Rio Grande do Norte, Rio Grande do Sul, São Paulo e Sergipe, além do Distrito

Federal, somando 801 municípios nesses estados (NEVES, 2005; MINISTÉRIO DA SAÚDE, 2014).

Figura 16 – Distribuição espacial dos hospedeiros intermediários de *Schistosoma mansoni* no Brasil



Fonte: SCHOLTE et al., 2012

B. straminea é a espécie mais bem adaptada e resistente à mudanças climáticas. Apesar de ser menos susceptível à infecção por *S. mansoni*, a espécie *B. straminea* é a mais abrangente no território brasileiro, com ocorrência em todos os estados, com exceção de do Amapá e Rondônia (Figura 16 - B) (CARVALHO et al., 2008; MINISTÉRIO DA SAÚDE, 2014).

A distribuição de *B. tenagophila* (Figura 16 - C) é mais restrita ao Sul e Sudeste do país, sendo a espécie responsável pela maioria dos casos nos estados de São Paulo e Santa Catarina e por focos isolados em Minas Gerais e Rio de Janeiro. Foi notificada em 562 municípios de 10 estados brasileiros (Bahia, Espírito Santo, Goiás, Mato Grosso do Sul,

Minas Gerais, Paraná, Rio de Janeiro, Rio Grande do Sul, Santa Catarina e São Paulo) (NEVES, 2005; CARVALHO et al., 2008; MINISTÉRIO DA SAÚDE, 2014).

Informações a respeito da distribuição dos moluscos hospedeiros intermediários de *S. mansoni* no Brasil (Figura 16 - D) são de grande importância, pois os registros de ocorrência desses animais fornecem um revelante aporte de conhecimento para o planejamento dos programas de controle da esquistossomose (CARVALHO et al., 2008)

2.7 CONTROLE DA ESQUISTOSSOMOSE

Desde a década de 50, a Organização Mundial de Saúde (OMS) tem estabelecido sucessivas diretrizes para o controle da esquistossomose em escala global (WHO, 1953, 1961, 1973, 1985, 1993, 2002). Na década de 50, o Comitê de Especialistas da OMS tinha foco nos estudos e pesquisas sobre a esquistossomose e sobre o estudo dos vetores transmissores da doença, além de aspectos geográficos, demográficos e socioeconômicos envolvidos na sua transmissão (WHO, 1953; BARBOSA et al., 2008).

Em 1961 e 1973, através de dois relatórios, o Comitê estabeleceu que a medida mais efetiva para o controle da doença seria o combate aos caramujos transmissores da parasitose através da utilização de agentes moluscicidas, já que o controle através da quimioterapia era considerado ineficaz, devido à ausência de drogas efetivas contra o parasito. Entretanto, também deixava estabelecida a importância de medidas associadas, como o tratamento de indivíduos infectados e a melhoria das condições de saneamento básico, para a erradicação da esquistossomose (WHO, 1961; 1973; BARBOSA et al., 2008).

A partir da década de 80, o Comitê de especialistas da OMS registrou uma importante mudança nas diretrizes de combate à esquistossomose, passando a ênfase para a quimioterapia e para as ações preventivas como saneamento e acesso à água potável. Essa nova abordagem colocava em destaque o hospedeiro humano ao reconhecer sua importância no sucesso e no fracasso dos programas de controle, sendo a aplicação de moluscicidas considerada como medida auxiliar de manutenção, com foco em áreas peridomiciliares de transmissão. Um dos fortes motivos da mudança de abordagem se deu em função do aumento de investimento da indústria farmacêutica em novas drogas com formulações mais seguras, além do crescente custo de agentes moluscicidas. Outras ações preventivas também foram citadas como o acesso à água potável, saneamento, educação em saúde e melhorias socioeconômicas (WHO, 1980; BARBOSA et al., 2008).

2.7.1 Tratamento quimioterápico e o praziquantel

No ano de 1917, após o tártaro emético ser utilizado pela primeira vez no tratamento da esquistossomose (CHRISTOPHERSON et al., 1918), assim como a utilização da emetina, em 1920, sendo considerada altamente tóxica, se observa um longo período de tempo para o início de novos relatos de outros agentes esquistossomicidas. A partir de 1960, entretanto, avanços significativos puderam ser observados no que concerne à descoberta de novas drogas esquistossomicidas, sendo introduzidos alguns compostos como o metrifonato, nitrofuranos, lucantone, niridazol, hicantone e oxamniquine. Entretanto, com exceção do oxaminiquine, todos os outros compostos apresentaram severos efeitos colaterais (LAGO et al., 2018).

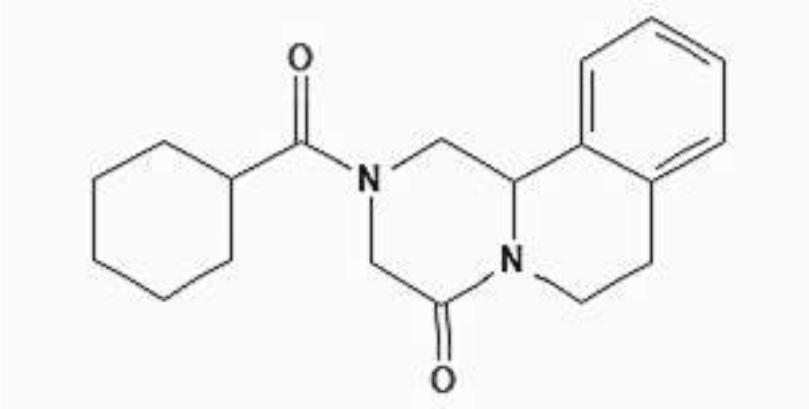
No final da década de 60, a atividade esquistossomicida do oxamniquine foi descrita, apresentando o fármaco com promissores resultados (LAGO et al., 2018). A toxidade do oxamniquine é considerada baixa, possuindo efeitos colaterais brandos (NEVES, 2005; KATZ, 2008). Possui efeito sobre vermes e fêmeas de *S. mansoni*, apresentando, também, atividade sobre fases imaturas do parasito, entretanto apresenta desvantagens como ineficácia na eliminação de outras espécies de *Schistosoma* de importância médica, bem como seu alto custo (CIOLI, 2000). Até a década de 90, o oxamniquine foi considerado o medicamento de primeira linha para o tratamento da esquistossomose, permanecendo em uso até 2010, sendo substituído pelo praziquantel (PZQ) até os dias atuais (VALENTIM et al., 2013; LAGO et al., 2018).

O Praziquantel foi descoberto na década de 70, através de esforços conjuntos da Merck e Bayer. O projeto inicial da Merck tinha por objetivo produzir e promover a descoberta de novos compostos tranquilizantes (SEUBERT; POHLKE; LOEBICH, 1977). Posteriormente, esses compostos foram testados pela Bayer com foco na atividade anti-helmíntica, sendo realizada uma triagem em mais de 400 compostos. Dentre estes, apenas o praziquantel foi selecionado, por apresentar segurança, eficácia, conveniência operacional e baixo preço (LAGO et al., 2018). Foi utilizado inicialmente para o tratamento de cestóides, apresentando atividade anti-helmíntica polivalente (KATZ, 2008).

O Praziquantel (Figura 17) é o nome genérico para 2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro 4H-pyrazino[2,1-a]isoquinoline- 4-one. Trata-se de um pó de coloração branca, de gosto amargo, estável em condições ambientes normais, e praticamente insolúvel em água, moderadamente solúvel em etanol e solúvel em solventes orgânicos como clorofórmio e dimetilsulfóxido (CIOLI; PICA-MATTOCCIA, 2003). As pastilhas de Praziquantel comercializadas são normalmente alongadas, contendo 600 mg do ingrediente

ativo. Sua preparação comercial é uma mistura de racemato composta por partes iguais de isômeros levógiros e dextrogiros, dos quais apenas o primeiro possui atividade esquistossomicida *in vitro* e *in vivo* (DOENHOFF; CIOLI; UTZINGER, 2008).

Figura 17 – Estrutura molecular do Praziquantel



Fonte: https://pubchem.ncbi.nlm.nih.gov

Wu et al. (1991), ao avaliarem pacientes tratados com o isômero biologicamente ativo de forma isolada e com a mistura racêmica, chegaram à conclusão de que a taxa de cura foi equivalente, entretanto os pacientes tratados apenas com o isômero biologicamente ativo para a atividade esquistossomicida apresentaram menos efeitos colaterais. Outros estudos já relatam que o maior responsável pela toxicidade do composto racêmico, bem como pelos efeitos colaterais, é o componente dextrógiro, sugerindo ser de extrema importância o desenvolvimento de novas formulações que contenham apenas o composto ativo (SUN et al., 2016; GREENBERG; DOENHOFF, 2017).

Sabe-se que o mecanismo molecular de ação do PZQ atua desestabilizando a homeostase do Ca^{2+} e a função neuromuscular do verme através de canais de Ca^{2+} voltagem-dependentes (GREENBERG, 2005), causando um comprometimento da estrutura e função do tegumento, espasmo e paralisia dos músculos subjacentes, culminando na eventual morte do parasita (LORSUWANNARAT et al. 2013). Alterações tegumentares também podem ser observadas após exposição dos vermes ao Praziquantel em um curto período de tempo, como o surgimento de bolhas, descamação e edema (AIRES et al., 2014; ARAÚJO et al., 2019) . Cepas de *Schistosoma* resistentes ao Praziquantel demonstraram, em ensaios de captação de Ca^{2+} e contração muscular, resultados atenuados em resposta ao Praziquantel, reforçando a

ligação entre os mecanismos destes ensaios e o mecanismo de ação do Praziquantel (CERQUEIRA et al., 2013).

Embora eficaz contra vermes adultos e considerado biosseguro e de baixo custo (BERTÃO et al. 2012), o PZQ, nas doses recomendadas, não apresenta eficácia terapêutica contra vermes jovens (3 a 4 semanas de desenvolvimento após a infecção), além de não prevenir a reinfecção. Sua formulação atual, na forma de racemato, contribui para o grande tamanho do comprimido e seu sabor amargo, o que dificulta a adesão ao tratamento, principalmente em crianças (GOUVEIA et al., 2018). Além disso, o tratamento em massa em países endêmicos para a erradicação da infecção é uma preocupação da comunidade médica e científica, pois há relatos de resistência e/ou tolerância das cepas de *Schistosoma* ao Praziquantel (BOTROS; BENNETT, 2007; WANG; WANG; LIANG, 2012; VALE et al. 2017). O potencial de resistência ao Praziquantel por *S. mansoni* foi relatado pela primeira vez em 1994 (FALLON; DOENHOFF, 1994). O estudo demonstrou que a droga aplicada sucessivas vezes em camundongos que foram expostos à sucessivas passagens em um pool de cercárias de quatro linhagens geográficas de *S. mansoni* eram menos sensíveis ao Praziquantel (BOTROS; BENNETT, 2007).

Diante deste cenário, é preocupante não possuir uma droga alternativa para uma doença em expansão que afeta milhões de indivíduos em todo o mundo e que possui tamanha importância em termos de saúde pública (BERTÃO et al., 2012). O monitoramento ativo da doença é necessário, ao mesmo tempo que esforços urgentes devem ser feitos para o desenvolvimento de novas drogas esquistossomicidas, visando uma maior eficácia no combate da esquistossomose (DOENHOFF et al., 2002).

2.7.2 Controle dos caramujos vetores e agentes moluscicidas

Durante quase toda a década de 70, a utilização de agentes moluscicidas apresentaram destaque nos programas de controle da esquistossomose, sobretudo porque as drogas disponíveis na época se mostravam ineficazes, causando inúmeros efeitos colaterais (WHO, 1993; 2001). Em tese, apesar do surgimento de uma droga eficiente na eliminação do parasito, a utilização de agentes moluscicidas constitui, ainda hoje, a única alternativa profilática em casos isolados, pois apresenta a possibilidade de interrupção temporária, porém imediata, da atividade dos focos de transmissão, pelo menos quando estes correspondem a coleções hídricas bem delimitadas, como lagoas de pequeno porte, poços ou valas de pequena extensão (TELES; CARVALHO, 2008).

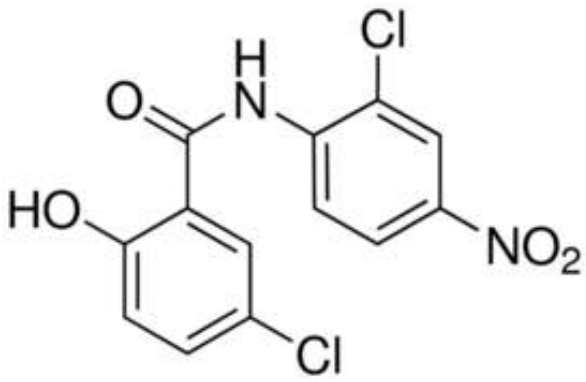
Estudos conduzidos em campo demonstraram uma redução significativa na transmissão da esquistossomose quando medidas de controle (utilização de moluscicidas e quimioterapia) eram aplicadas periodicamente em áreas endêmicas por alguns anos (KATZ, 1998). Pesquisas realizadas em meados da década de 70, com foco na espécie *B. straminea*, mostraram que houve uma diminuição significativa em número desses vetores nos focos de transmissão bem como das taxas de infecção natural desses animais, além da redução dos índices de infecção humana (prevalência, incidência e intensidade) nas áreas tratadas com moluscicidas, quando comparadas com áreas não tratadas (BARBOSA; COSTA, 1981; BARBOSA et al., 2008).

Um composto moluscicida ideal deve eliminar os caramujos em todas as fases de seu ciclo de vida, desde a fase embrionária até a fase adulta. Deve apresentar baixo custo, ser estável, de fácil transporte e aplicação, ausência de risco na manipulação, ser seletivo aos moluscos, inofensiva ao homem, animais não-alvo e plantas (WHO, 1965, 1985; RAPADO, 2012).

Diversos produtos já foram utilizados na tentativa de se encontrar um composto moluscicida ideal para o combate dos caramujos vetores, como por exemplo a cal extinta, o sulfato de cobre, o pentaclorofenato de sódio, a tritilmorfolina e a niclosamida, todos estes comprovadamente tóxicos ao meio ambiente. No entanto, o único agente moluscicida utilizado em campanhas de controle nacional, tendo sua recomendação endossada pela OMS, é a niclosamida, produzida comercialmente pela Bayer (WHO, 1965; BARBOSA et al., 2008).

A niclosamida (2′,5-dichloro-4′-nitrosalicylanilide) é um sólido amarelado, insípido e inodoro, com peso molecular de 327, 1 e fómula química de $C_{13}H_8Cl_2N_2O_4$ (Figura 18). É o componente ativo presente em 25% da formulação do Bayluscide® EC 250, já seu sal de etanolamina representa 75% da formulação do Bayluscide® WP70. Possui bioatividade não só como moluscicida, mas também como anti-helmíntico, com foco na classe Cestoda, estando presente nas preparações de Yomesan®, Mansonil® e Lintex ®, utilizados na medicina humana e veterinária no tratamento da teníase (ANDREWS; THYSSEN; LORKE, 1983).

Figura 18 – Estrutura molecular da niclosamida



Fonte: https://www.sigmaaldrich.com

A niclosamida é considerada um potente desacolpador, tendo o potencial de demonstrar bioatividade sobre mitocôndrias isoladas de mamíferos e insetos em concentrações próximas de 1 nM, além de ser quase 1000 vezes mais ativo que o 2,4-DNP, um xenobiótico utilizado na fabricação de tintas, herbicidas, pesticidas e explosivos. Possui um comportamento típico dos demais desacopladores, estimulando a respiração mitocondrial em baixas concentrações, inibindo-a a medida que a concentração é aumentada (HOLLINGWORTH et al., 2001).

Possui elevada toxicidade para moluscos, em concentrações próximas a 1 mg/L, causando 100% de mortalidade em até 8 horas de exposição para os gêneros *Biomphalaria* e *Bulinus* (REY, 2001). Apesar de ser eficiente na eliminação dos moluscos em suas diferentes fases de desenvolvimento, apresenta desvantagens em sua utilização como a toxicidade elevada para espécies não-alvo, como peixes, plantas e outros organismos aquáticos em uso prolongado, possui custo elevado, se decompõe sob luz solar, é irritante para a pele e mucosas, além de não prevenir a recolonização em áreas tratadas (RAPADO, 2012; OLIVEIRA-FILHO; PAUMGARTTEN, 2000; ABREU; GOULART; OLIVEIRA BRETT, 2002; ANDREWS; THYSSEN; LORKE, 1983; LOKER, 1990).

2.8 PRODUTOS NATURAIS APLICADOS AO CONTROLE DA ESQUISTOSSOMOSE

A biodiversidade é uma fonte valiosa de novidades em termos de moléculas bioativas, produzindo uma variedade extraordinária de métabólitos secundários (DAR et al., 2017). A utilização de produtos naturais como medicamentos foi descrita ao longo da história sob a forma de medicamentos tradicionais, remédios, poções e óleos, entretanto, muitos desses

produtos naturais bioativos ainda não foram identificados (DIAS et al., 2012). Por um longo período, produtos naturais e seus extratos foram os únicos medicamentos disponíveis para a humanidade. A fonte de conhecimento sobre o uso de produtos naturais a partir de plantas medicinais é o resultado do homem experimentar por tentativa e erro, por centenas de séculos, geralmente através de ensaios de palatabilidade, resultando muitas vezes em mortes prematuras, de buscar alimentos disponíveis para o tratamento de doenças (DIAS; URBAN; ROESSNER, 2012; DAR et al., 2017).

Os produtos naturais tem uma ampla variedade e diversidade de estruturas químicas, sendo sua eficácia relacionada com a complexidade das suas propriedades estereoquímicas bem organizadas, oferecendo muitas vantagens em termos de eficiência e seletividade de alvos moleculares (YUAN et al., 2016). Na última década, algumas grandes empresas farmacêuticas, focando na química combinatória, deixaram os produtos naturais de lado e tentaram preencher essa lacuna com um grande número de moléculas sintéticas. Infelizmente, a química empregada não criou o que se esperava de moléculas suficientemente diversas ou farmacologicamente ativas. Recentemente notou-se a volta do crescimento do interesse da indústria farmacêutica nos produtos naturais, levando em conta agora a síntese orientada para a diversidade, através do mimetismo de estruturas oriundas destes produtos, potencializando a descoberta de novos medicamentos (ZHANG; DEMAIN, 2005; DIAS; URBAN; ROESSNER, 2012).

Exemplos bem sucedidos de desenvolvimento de drogas a partir de produtos naturais são a Ivermectina e a Artemisina. A primeira destas, descoberta por William C. Campbell e Satoshi Omura, ajudou a reduzir radicalmente a incidência de oncocercose e filariose linfática. A Artemisina, descoberta pela peaquisadora Tu Youyou, é utilizada com sucesso e de forma ampla para o tratamento da malária. Ambas as descoberta concederam aos três pesquisadores o Prêmio Nobel de Medicina e Fisiologia de 2015, demonstrando como a pesquisa baseada em produtos naturais pode ser significativa no desenvolvimento de novos medicamentos, impactando positivamente a qualidade de vida da população (CRAGG; NEWMAN, 2013; MUSCHIETTI et al., 2013; SHEN, 2015).

2.8.1 Esquistossomicidas de origem natural

Diversos extratos ou constituintes bioativos de organismos vivos tem sido utilizados na medicina popular em todo o mundo, visando o combate às doenças parasitárias, incluindo a esquistossomose, e nas últimas décadas, os produtos naturais tem atraído um interesse

renovado (MORAES, 2012). Nesse contexto, diversas espécies de plantas se tornaram alvo de estudo em triagem *in vitro* e *in vivo*, visando encontrar preparações com maior atividade ou o isolamento de biomoléculas ativas, que possam conduzir ao estabelecimento de novos fármacos anti-helmínticos (CASTRO et al., 2013). Um esforço considerável tem sido observado na busca e desenvolvimento de compostos esquistossomicidas. Neste cenário, diversos compostos de origem natural com propriedades esquistossomicidas promissoras foram identificados (BERTÃO et al., 2012; AIRES, et al., 2014; GUIMARÃES et al., 2015; ARAÚJO et al., 2019).

A eficácia destes novos compostos pode ser definida utilizando-se três estratégias 1) A curativa, matando o verme adulto, 2) A profilática, matando as formas imaturas do verme, 3) A supressora, inibindo a postura de ovos. Desta forma, vários parâmetros como análise de motilidade, alterações tegumentares e oviposição, são frequentemente avaliados em triagens de novos compostos esquistossomicidas como indicadores de bioatividade (MORAES, 2012).

A maioria dos estudos de atividade esquistossomicida de preparações de origem natural se inciam com o *screening in vitro* destes compostos, onde são avaliados seus efeitos sobre diferentes fases evolutivas do parasito como as fases de cercaria, esquistossômulo, e vermes jovens e adultos (CASTRO et al., 2013), sendo de grande importância a associação com testes citotoxicidade, onde a especificidade do composto é analisada. As análises de viabilidade celular *in vitro* e *ex vivo* utilizadas em ensaios de citotoxicidade nas diversas áreas biomédicas englobam linhagens celulares variadas como, por exemplo, PBMCs (Células Mononucleares de Sangue Periférico) (ALBUQUERQUE et al., 2014; ARAÚJO et al., 2019), fibroblastos (UDDIN; GRICE; TIRALONGO, 2011; AYAZ et al., 2016), macrófagos (TEMPONE et al., 2005; AHMED et al., 2011; OLIVEIRA et al., 2014) e hemácias (TOLEDO et al., 2011; EVANS et al., 2013) para a triagem toxicológica de vários compostos químicos. Estes testes de citotoxicidade presentam vantagens devido à simplicidade da análise, rapidez, baixo custo e a possibilidade de abranger um elevado número de amostras de uma só vez. Além disso, ensaios de viabilidade celular e citotoxicidade tem como base a avaliação de diversas funções celulares como permeabilidade membranar, atividade enzimática, aderência celular, produção de ATP e atividade de captação de nucleotídeos (ISHIYAMA et al., 1996; ASLANTÜRK, 2018).

Dentro desta ótica, diversos grupos químicos foram testados para verificação quanto sua atividade esquistossomicida como: Terpenos (epóxido de limoneno, rotundifolona, artemisina e derivados, nerolidol) (UTZINGER et al., 2001; MATOS-ROCHA et a., 2013, MORAES, et al., 2013; LIU; WU; LIANG, 2014), alcalóides (pirplatina, piperamida,

sanguinarina, epiisopiloturina) (MORAES et al., 2011, MORAES et al., 2012; VERAS et al., 2012; ZHANG; COULTAS, 2013; CARRARA et al., 2014; GUIMARÃES et al., 2014), peptídeos (ciclotídeos Kalata B1 e Kalata B2) (MALAGÓN et al., 2013), e compostos fenólicos, englobando as quinonas (pumblagin e β-lapachona) (ZHANG; COULTAS, 2013; LORSUWANNARAT et al., 2013; AIRES et al., 2014; AIRES et al., 2014), flavonoides (quercetina, kaempferol, sativan) (BRAGUINE et al., 2012; CUNHA et al., 2012; XIAO et al., 2014), neolignanas (licarina A) (PEREIRA et al., 2011) e dibenzofuranos (SALLOUM, et al., 2012; ARAÚJO et al., 2019).

2.8.2 Moluscicidas de origem natural e ensaios ecotoxicológicos

A dificuldade em controlar a esquistossomose associada aos altos custos e elevada toxicidade dos moluscicidas sintéticos, bem como a busca por substâncias biodegradáveis e inócuas ao meio ambiente e ao homem, vem estimulado as pesquisas científicas direcionadas à descoberta de novos agentes moluscicidas de origem natural (MIYASATO et al., 2012; MARTINS et al., 2014; ROCHA-FILHO et al., 2015; MARTINS et al., 2017; SILVA et al., 2018; ARAÚJO et al., 2018).

A busca por compostos moluscicidas de origem natural surgiu na década de 30, após a sugestão do cultivo de *Balanites aegyptiaca* L. (Balanitaceae), espécie africana nativa, próximo a criadouros de caramujos vetores da esquistossomose no Sudão. Notou-se que os frutos que caíam nas águas do criadouro inibiam o crescimento da população de moluscos ali presentes. Este fato impulsionou os estudos realizados posteriormente que comprovaram, além dos frutos, outros tecidos da *B. aegyptiaca* (raízes, cascas e ramos), possuíam, de fato, atividade moluscicida. O provável componente bioativo se trata de uma saponina, cujo potencial de letalidade atinge também miracídios e cercárias de *Schistosoma*, peixes e girinos (ARCHIBALD, 1933; SILVA et al., 2008; GARDIOLI, 2014).

Mais de 42 compostos moluscicidas já foram isolados de espécies vegetais e diversos estudos sugerem que a atividade tóxica dos agentes moluscicidas de origem vegetal está relacionada à presença de metabólitos secundários como flavonoides, alcaloides, terpenoides, saponinas, taninos, esteroides, dentre vários outros (WHO, 1983; LOPES et al., 2011; CANTANHEDE at al., 2010). Ademais, outros compostos não vegetais também vem ganhando destaque como possíveis agentes moluscicidas, como é o caso de metabólitos produzidos por bactérias, fungos, esponjas e líquens (ALI et al., 2010; ANGELO; VILAS-

BÔAS; CASTRO-GÓMEZ, 2010; GUO et al., 2010; MIYASATO et al., 2012; MARTINS at al., 2014).

De acordo com a OMS, são agentes moluscicidas reconhecidos aqueles que, se purificados, apresentarem níveis acima de 90% de letalidade sobre os caramujos em concentrações iguais ou inferiores a 20 µg mL^{-1} no período de até 24 horas. Caso seja um composto não purificado, estando em sua forma bruta, é reconhecido pela OMS como moluscicida caso apresente níveis acima de 90% de letalidade em concentrações iguais ou inferiores a 100 µg mL^{-1} no período de até 24 horas. É recomendado, ainda, que o produto natural seja originário da região endêmica onde terá aplicação, acessível à população, de baixo custo, de curto ciclo reprodutivo, estável sob variações de pH, temperatura e luminosidade, e biodegradável (KLOOS; MCCULLOUGH, 1982; WHO, 1983).

Um agente moluscicida ideal deve possuir alta especificidade sobre a espécie alvo, sendo inócuo para organismos não-alvo. Desta forma, faz-se necessária a avaliação destes agentes por meio de ensaios ecotoxicológicos, nos quais se utilizam organismos sensíveis a uma diversidade de agentes químicos. O nível de sensibilidade do organismo-teste ao agente potenciamente tóxico deve ser constante, visando à obtenção de resultados precisos, e boas repetilidade e reprodutibilidade (SUNDFELD, 2010).

Por meio destes ensaios é possível avaliar os efeitos causados ao organismo-teste através da exposição às variadas concentrações da substância potencialmente tóxica a ser avaliada, por um período de tempo preestabelecido. Podem ser avaliados efeitos agudos, observando critérios de letalidade e motilidade, e os efeitos crônicos, onde as variáveis mais comuns são alterações de crescimento, reprodução e sobrevivência (MAFFAZZIOLI, 2011). São exemplos de análises ecotoxicológicas o bioensaio de toxicidade aguda com o microcrustáceo *Artemia salina* (Figura 19) (CANSIAN et al., 2017; SILVA et al., 2018), o ensaio de toxicidade aguda com a bactéria luminescente *Vibrio fischeri* (Sistema Microtox) (VENTURA et al., 2012; ABBAS et al., 2018), bioensaio de toxicidade crônica com o microcrustáceo *Ceriodaphnia dubia* (LAMICHHANE et al., 2013; SOUZA et al., 2018) e o teste de mutação reversa (teste de Ames) utilizando *Salmonella* (RESENDE et al., 2012; CHAO et al., 2018).

Dentre estes bioensaios, a avaliação realizada com *Artemia salina*, devido a sua ampla distribuição geográfica, fácil manutenção laboratorial, curto ciclo de vida, se destaca sendo considerado um biensaio simples, de fácil reprodução e boa confiabilidade (NUNES et al., 2006; ARAÚJO et al., 2016).

2.9 LÍQUENS

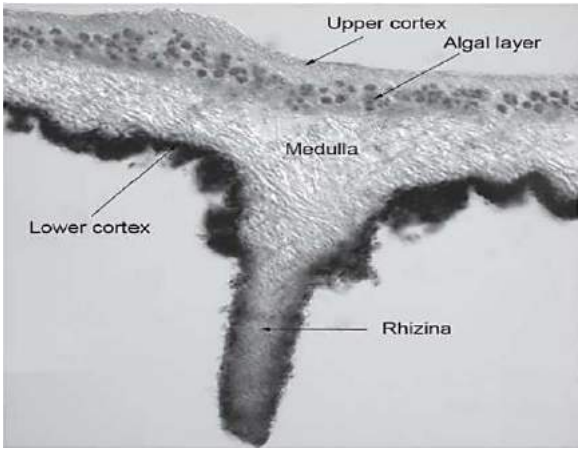
Desde a década de 1860, os líquens, também chamados de fungos liquenizados, são classicamente descritos como associações simbióticas complexas e estáveis entre um micobionte, geralmente um ascomiceto, e ao menos um componente fotoautotrófico (algas ou cianobactérias) (BRIGHTMAN; SEAWARD, 1977; BENATTI; MARCELLI, 2007; ZÚÑIGA et al., 2017). Juntos, esses organismos formam talos de diferentes morfologias, que não se assemelham a nenhum dos simbiontes isoladamente (SPRIBILLE et al., 2016). Porém, recentemente foi descrita a presença de outros componentes fazendo parte dessa associação em diversas espécies. A definição clássica dos líquens foi atualizada quando um segundo micobionte foi encontrado incorporado ao córtex liquênico, as leveduras de basidiomicetos, estando sua presença diretamente relacionada com variações fenotípicas (ZÚÑIGA et al., 2017; SPRIBILLE et al., 2016). Entretanto, antes da descoberta das leveduras de basidiomicetos como parte integrante do córtex liquênico, algumas espécies de bactérias também foram encontradas em íntima associação com o talo liquênico (CARDINALE et al., 2008; GRUBE et al., 2009; HODKINSON et al., 2011). Desta forma, a definição de líquen como um organismo simbionte bipartido ou tripartido foi modificada, sendo agora considerado como um simbionte multiespécie (ZÚÑIGA et al., 2017).

Os fungos formadores de líquens estão subdivididos em ascomicetos (98% dos líquens), basidiomicetos, deuteromicetos e mastigomicetos. Os fotobiontes procariontes estão subdivididos entre os gêneros *Nostoc*, *Gleocapsa*, *Scytonema*, *Stigonema*, *Chroococcus*, *Hyella*, *Calothrix* e *Dichotrix*. As algas compreendem as *Chlorophyta*, *Xanthophyta* e *Phaeophyta* (PODTEROB, 2008). Fotobiontes eucariotos podem ser também denominados de ficobiontes (correspondendo a 90% dos líquens), enquanto os fotobiontes procariontes (cianobactérias) são denominados de cianobiontes (correspondendo a 10% dos líquens) (RANKOVIĆ; KOSANIĆ, 2015).

A maioria dos líquens possui um talo estratificado (Figura 19), talos heterômeros, constituídos de até 4 camadas principais: (1) o córtex superior, que consiste em uma aglutinação de hifas fúngicas entrelaçadas, as quais fornecem proteção para a camada seguinte. (2) a camada algal, que representa cerca de 7% do volume do talo, onde os fotobiontes se inserem de forma frouxa. (3) a medula, onde as hifas estão parcialmente compactadas, e juntas formam boa parte do talo liquênico. É o local onde ocorrem as trocas de gases e onde se encontram a maioria dos ácidos liquênicos. (4) Córtex inferior, que é anatomicamente semelhante ao córtex superior, onde podem surgir as rizinas (estruturas de

fixação), a depender da espécie liquênica. Em talos com distribuição uniforme, onde há pouca diferenciação entre os estratos ou camadas, o talo é denominado homômero, sendo composto por uma aglutinado frouxo de hifas com células de algas, igualmente distribuídas ao longo do talo (JAHNS, 1973; TAYLOR; KRINGS; TAYLOR, 2014).

Figura 19 – Representação de um talo liquênico heterômero



Fonte: MOLNÁR; FARKAS (2010)

A morfologia dos líquens é extremamente diversificada, além de possuírem uma imensa variedade de cores , incluindo laranja, amarelo, vermelho, verde, cinza, marrom e preto (ROMAGNI; DAYAN, 2002). Podem variar em tamanho, desde indivíduos muito pequenos (1 mm^2) até estruturas caídas com mais de um metro de comprimento (NASH III, 1996). A morfologia do talo liquênico é primariamente determinada pelo componente fúngico. Porém, as características morfológicas do talo só adquirem definição após o processo de simbiose, não se assemelhando ao componente fúngico de forma isolada. A morfologia geral dos talos liquênicos pode ser subdividida em três categorias principais (Figura 20): crostosos, foliosos e fruticosos (ROMAGNI; DAYAN, 2002).

Figura 20 – Tipos de talos liquênicos. A - Talo crostoso. B – Talo folioso. C – Talo fruticoso

Fonte: A – http://www.learnaboutnature.com . B - http://www.drmgoeswild.com .
C - https://www.teara.govt.nz . Acesso em 10.04.2018.

Os líquens crostosos possuem o talo aderido firmemente ao substrato. A maior parte dos liquens crostosos cresce diretamente na superfície do substrato e é referido como episubstrático, enquanto uma pequena minoria cresce dentro do substrato chamado endossubstrático. O talo episubstrático é constituído por um aderente de crescimento em forma de crosta ou ligado ao substrato por todo o seu lado por hifas e não pode ser desprendido sem destruição (SHUKLA; UPRETI; BAJPAI, 2014). Eles geralmente são espécies sucessionais primárias que sobrevivem em áreas inóspitas e, em muitos casos, os fracos ácidos liquênicos produzidos decompõem lentamente as rochas para espécies sucessionais posteriores (ROMAGNI; DAYAN, 2002)

Os líquens foliosos são estruturas foliares bidimensionais, tipicamente achatados dorsoventralmente, espalhando-se e expandindo-se horizontalmente para as laterais. Este grupo exibe uma grande variedade de diversidade e tamanho de talo. A superfície inferior desses liquens costuma ter algum tipo de estrutura de fixação, como rizinas ou cílios (ROMAGNI; DAYAN, 2002; SHUKLA; UPRETI; BAJPAI, 2014).

Os líquens fruticosos são organismos tridimensionais que se destacam do seu substrato e são caracterizados como semelhantes a pêlos, arbustos, musgos ou pendentes. Esses liquens, por definição, têm uma alta razão superfície/volume que resulta em um ciclo mais rápido de secagem e umedecimento. Eles são
preferencialmente encontrados em climas úmidos ou em climas áridos onde a precipitação oculta (névoa) é comum (ROMAGNI; DAYAN, 2002).

Líquens podem colonizar uma vasta gama de habitats, desde regiões do ártico até regiões tropicais, desde habitats aquáticos até locais de elevada altitude (MÜLLER, 2001; MOLNÁR; FARKAS, 2010). Ocorrem na maioria dos ecossistemas terrestres do mundo. Em muitos ecossistemas polares e subpolares, os líquens são considerados os seres autótrofos dominantes. Desempenham importantes papeis em ecossistemas alpinos, costeiros e florestais, como as florestas temperadas do hemisfério sul e a taiga no hemisfério norte (NASH III, 2008). Utilizam como substratos os mais diferentes tipos de estruturas e materiais como rochas, solo, em troncos e arbustos de árvores, sobre folhas e carapaças de animais, e sobre várias superfícies artificiais como pedaços de madeira, couro, vidro, metais, concreto, tijolos, borracha e plásticos (BRIGHTMAN; SEAWARD, 1977; SEAWARD, 2008).

A relação de simbiose entre um micobionte e fotobionte, resultando na liquenização, permite que tanto a alga quanto o fungo consigam sobreviver em circunstâncias que não suportariam como organismos isolados. Por exemplo, muitos líquens comuns ocorrem na superfície de rochas, em plena luz solar. Tais habitats são muito expostos e pobres em

nutrientes. Claramente, nota-se que seria um local de difícil colonização por algas, geralmente organismos aquáticos, ou fungos, que necessitam de habitats que forneçam melhores condições nutricionais (STEPHENSON, 2010).

A utilização dos líquens é diversa e antiga, foram relatadas suas utilizações para diversos fins como a nutrição de homens e animais, como corantes, remédios, perfumes, indicadores de pH, matéria prima para tecidos, dentre outras (MITROVIĆ et al., 2011). Diferentes gêneros de líquens são usados na cura de diversas doenças como febres, diarreia, doenças de pele, epilepsia, convulsões, asma, bronquite, inflamações, lepra, cálculos de vesícula, etc. (SHUKLA; JOSHI; RAWAT, 2010).

2.9.1 Os gêneros *Canoparmelia, Cladia e Ramalina*

O gênero *Canoparmelia,* pertencente à família Parmeliaceae, compreende espécies liquênicas cosmopolitas, ocorrendo, principalmente, nas Américas e África (CUNHA; MARCELLI; PEREIRA, 2015). Opta pelo desenvolvimento onde existe maior luminosidade e baixa umidade atmosférica (JUNGBLUTH, 2006). Cerca de 45 espécies foram descritas em todo o mundo, se dispersando, principalmente, em regiões tropicais e subtropicais (NASH III; ELIX, 2002).

A *Canoparmelia texana* é um líquen folioso comumente encontrado nos cerrados e áreas urbanas do Brasil. Geralmente utiliza cascas de árvores como substrato, possui rizinas simples que alcançam as margem dos lobos, e coloração cinza claro ou cinza esverdeado ou, raramente, amarelo esverdeado. Tem sido alvo de estudos devido ao seu potencial como bioindicador ambiental da poluição do ar (SAIKI et al., 2003; BARBOSA et al., 2010; CUNHA; MARCELLI; PEREIRA, 2015). Apresenta os métabólitos atranorina no córtex superior e ácido divaricático na medula (ELIX; JOHNSTON; VERDON, 1986).

O gênero *Cladia,* Cladoniaceae (Lecanorales, Ascomycota), compreende 14 espécies, onde a maioria ocorre na Austrália. Uma grande variedade do gênero é observada na Tasmânia, onde 11 espécies são relatadas. O gênero também possui representantes na Nova Zelândia e América do Sul. As espécies de *Cladia* possuem uma notável variedade química e morfológica, dificultando a determinação das espécies. Os representantes deste gênero são caracterizados pela presença de inúmeras perfurações distribuídas no talo secundário, o qual é ereto, ramificado, com medula de coloração branca ou marrom escuro. O gênero também apresenta uma variedade de metabólitos secundários, especialmente depsídeos, depsidonas, ácido úsnico, ácidos graxos e triterpenóides. A espécie *Cladia aggregata* tem ampla

distribuição, ocorrendo no sudeste da Ásia, Coréia, Índia, África do Sul, e Américas Central e Sul (FILSON, 1981; KANTVILAS; ELIX; JOHNSTON; VERDON, 1986; AHTI, 2000; HUR et al., 2004; LUMBSCH et al., 2010).

No gênero *Ramalina*, o talo é fruticoso e, em espécies brasileiras, seus comportamentos são comumente cespitoso (formando tufos densos e aglomerados), decubente (os ramos crescem horizontalmente no chão e as extremidades se colocam em posição vertical) e pendular (os quais se fixam no substrato e crescem pendurados). O talo é composto por ramificações que crescem a partir da base liquênica. Os ramos são, em grande parte, dorsiventrais ou cilíndricos. Ramos dorsiventrais, em sua grande maioria, possuem as superfícies dorsal e ventral bem distintas, geralmente apresentando diferença de coloração. Este tipo de talo é encontrado nas espécies *R. asahinae*, *R. aspera*, *R. cochlearis*, *R. complanata*, *R. grumosa*, *R. prolifera*, *R. puiggarii* e *R. subpollinaria*. Os ramos cilíndricos geralmente são circulares em corte transversal, sendo encontrados nas espécies de *R. sorediosa* e alguns morfotipos de *R. sprengelli* ou *R. usnea* (KASHIWADANI; KALB, 1993).

Geralmente as espécies do gênero escolhem como substrato o tronco ou galhos de árvores. Nos biomas Cerrado e Caatinga brasileiros, encontramos as espécies *R. aspera, R. complanata, R. peruviana, R. sorediosa, R. subpollinaria* e *R. usnea*. No Brasil, a ocorrência de *R. aspera* já foi relatada nos estados de Minas Gerais, Mato Grosso do Sul, Rio Grande do Sul, Santa Catarina, São Paulo, Sergipe, Pernambuco, Alagoas. São relatadas duas raças químicas: a primeira apresenta os ácidos liquênicos divaricático e úsnico, já a segunda os ácidos úsnico e criptoclorofaeico (GUMBOSKI, 2016).

2.10 METABÓLITOS LIQUÊNICOS

Os metabólitos liquênicos podem ser divididos em dois grandes grupos: os metabólitos primários e os metabólitos secundários. Os primeiros são representados por proteínas, lipídeos, carboidratos e outros compostos orgânicos relacionados ao metabolismo e composição da estrutura do líquen. Os metabólitos secundários, também denominados "substâncias liquências", são produzidos em sua maioria pelo componente micobionte, sendo depositadas na superfície das hifas do fungo, seja na forma amorfa ou na forma de cristais (MITROVIĆ et al., 2011; HONDA; VILEGAS, 1998).

Os metabólitos liquênicos representam cerca de 5 a 10% do peso seco do líquen, podendo alcançar percentuais acima de 30% (MUGGIA; SCHMITT; GRUBE, 2009; BACKOROVÁ et al., 2012; TURK et al., 2003; ELIX, 1996). Estes compostos apresentam

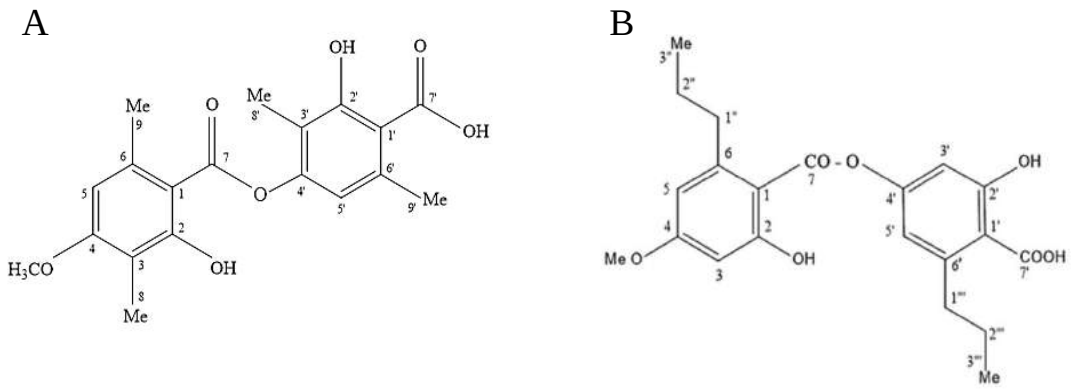
um papel de proteção importante para a estrutura liquênica, protegendo-os tanto contra fatores abióticos como a herbivoria e competição, como contra a radiação UV. É estimado que mais de 50% dos líquens produzam ácidos liquênicos com propriedades antimicrobianas. Essa característica pode ter um papel fundamental na ecologia e na dinâmica ecossistemática geral dos líquens (ROMAGNI; DAYAN, 2002).

A maioria dos metabólitos secundários liquênicos são compostos fenólicos que, de acordo com suas estruturas químicas, podem ser classificados em dibenzofuranos e ácido úsnico, depsídeos (ácido barbático e ácido divaricático), depsidonas (ácido salazínico), depsonas (ácido picroliquênico), lactonas (ácido protoliquesterínico e ácido nefrosterínico), quinonas (parietina) e derivados do ácido pulvínico (BOUSTIE; GRUBE, 2005).

Os líquens possuem diversas vias biossintéticas para a produção de suas substâncias liquênicas, sendo as principais: via do acetato polimalonato, via do ácido chiquímico e via do ácido mevalônico. A via do acetato polimalonato dá origem a maioria dos metabólitos secundários liquênicos, sendo responsável pela síntese de depsídeos, depsidonas, depsonas, dibenzofuranos, ácido úsnico, cromonas, xantonas e antraquinonas. Estes metabólitos, produzidos pelo micobionte, desempenham um importante papel na associação simbiótica. A via do ácido chiquímico dá origem às terfenilquinonas (ácidos aromáticos) e ao ácido pulvínico. Essas classes de compostos são amplamente encontradas em líquens da família Stictaceae e são geralmente obtidos pela fusão de duas unidades fenilpiruvato (BOUSTIE; GRUBE, 2005; MUGGIA; SCHMITT; GRUBE, 2009; MITROVIĆ et al., 2011). A via do ácido mevalônico origina os diterpenos, triterpenos e esteroides. Esteróis e terpenos não são comumente encontrados em espécies liquênicas, sendo mais abundantes em espécies vegetais superiores. O ergosterol, fitosterol e o β-sitosterol são exemplos de esteróis que ocorrem em líquens. Já os triterpenos são encontrados com mais frequência em espécies liquênicas, sendo a zeorina o triterpeno mais conhecido e encontrado (HONDA; VILEGAS, 1998).

Os depsídeos são formados pela esterificação de duas unidades fenólicas derivadas do orcinol (carboxila da posição 1 da primeira unidade com a hidroxila da posição 4’ ou da posição 3’ da segunda unidade), podendo resultar em compostos denominados para-depsídeos ou meta-depsídeos (HONDA; VILEGAS, 1998). Exemplos de depsídeos são os ácidos divaricático e barbático, os quais tem sua estrutura química demonstrada na Figura 21.

Figura 21 – Estrutura molecular dos ácidos (A) barbático e (B) divaricático



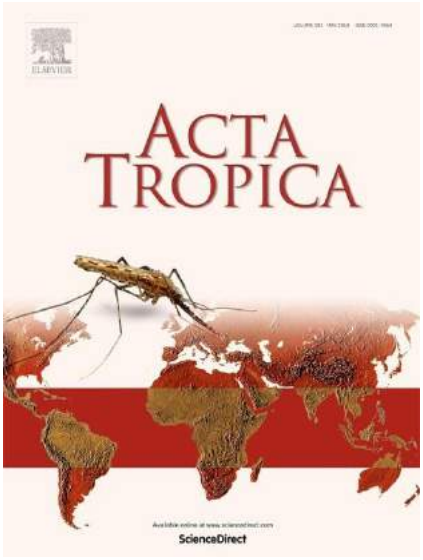
Fonte: Autor (2019)

Diversos estudos reportam atividades biológicas promissoras dos ácidos barbático e divaricático. O ácido divaricático é citado na literatura por possuir atividade antitumoral sobre células cancerígenas de próstata, adenocarcinoma renal, adenocarcinoma de mama, melanoma, adenocarcinoma colorretal, carcinoma laríngeo e lipoma (RUSSO et al., 2012; BOGO, 2012), atividade antimicrobiana (MOURA, 2008; RIBEIRO et al., 2002), atuar como inibidor enzimático (SANKAWA et al., 1982) e possuir atividade moluscicida e cercaricida (SILVA et al., 2018). Em estudos desenvolvidos com o ácido barbático, já foram reportadas as atividades antimicrobianas, antioxidante, inseticida, herbicida, antitumoral, moluscicida e cercaricida (RANKOVIĆ; KOSANIĆ 2015; VERMA; BEHERA, 2015; MARTINS et al. 2016; 2017; 2018).

Levando-se em consideração o alto potencial biológico dos ácidos divaricático e barbático, e a necessidade da busca de novas drogas esquistossomicidas e agentes moluscicidas, 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 objetos de estudo o ácido divaricático (obtido de *R. aspera* e *C. texana*) e o ácido barbático (obtido de *C. aggregata*).

3 ARTIGO 1 - TOXICOLOGICAL EFFECTS OF *Ramalina aspera* (LICHEN) ON *Biomphalaria glabrata* SNAILS AND *Schistosoma mansoni* CERCARIAE

ARTIGO PUBLICADO NO PERIÓDICO



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Toxicological effects of *Ramalina aspera* (lichen) on *Biomphalaria glabrata* snails and *Schistosoma mansoni* cercariae

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ABSTRACT

In this study, the molluscicidal activities against *Biomphalaria glabrata* and cercaricidal activities against *Schistosoma mansoni* of the ether extract of *Ramalina aspera* were evaluated. Additionally, toxicity parameters were evaluated at sublethal doses in terms of the influence of the extract on the fertility and fecundity of snails, as well as morphological alterations and quantification of their immunological cells. A test with *Artemia salina* was also carried out, in order to verify the environmental toxicity of the compound. The ether extract of *R. aspera*, in which divaricatic acid was identified as the major compound, demonstrated molluscicidal activity at low concentrations against both embryos (LC_{90} of 22.78, 24.23, 16.63 and 16.03 μg mL^{-1} for the gastrula, blastula, trochophore and veliger, respectively) and against adult snails (LC_{90} of 8.66 μg mL^{-1}), after 24 h of exposure. At the sublethal doses, it was possible to observe a decrease in fecundity and quantitative and morphological changes in the defense cells of the exposed snails. In addition, the extract of *R. aspera* showed a cercaricidal effect on *S. mansoni* from the concentration of 5.0 μg mL^{-1}, while showing low toxicity to *Artemia salina*. The ether extract of *R. aspera* demonstrated effective molluscicidal activity on embryos and adult snails of the species *B. glabrata*, cercariae of *S. mansoni*, and presenting low toxicity on *Artemia salina*. In this way, it could be considered a promising compound in the development of future molluscicidal and cercaricidal agents, thus helping to combat schistosomiasis.

1. Introduction

Among human parasites, schistosomiasis, a parasitic infectious disease caused by trematode worms of the genus *Schistosoma*, occupies second place, behind only malaria, in terms of public health importance (Colley et al., 2014; Mutengo et al., 2014). Its transmission to humans occurs through the penetration of cercariae, the larval stage of the parasite, through contact of the skin with contaminated water (Gryssels et al., 2006; World Health Organization, 2018). It is estimated that approximately 261 million people are infected worldwide and 659 million are at risk of contracting the disease (WHO, 2018). The parasite has a heteroxenous life cycle, having as intermediate hosts snails of the genera *Bulinus*, *Oncomelania*, and *Biomphalaria* (Gryssels et al., 2006; Roquis et al., 2014). In the Americas and Africa, the genus *Biomphalaria* is responsible for the transmission of *Schistosoma mansoni*, one of the agents of intestinal schistosomiasis (Colley et al., 2014).

Strategies for the control of schistosomiasis include the use of molluscicidal agents for the control of intermediate hosts, along with mass drug administration, sanitation, and health education (Kariuki et al., 2013; Inobaya et al., 2014). Currently, the only molluscicide recommended by the World Health Organization is Niclosamide (Bayluscide, Bayer) (WHO, 2002), a synthetic substance that, although efficient in eliminating vector snails, is expensive in its application, decomposes under sunlight and presents a high level of toxicity to non-

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target organisms such as fish, amphibians and plants (Oliveira-Filho and Paumgartten, 2000; Faria et al., 2018). The development molluscicides of natural origin, non-toxic to the environment and effective in combating vector mollusks and the infectious larval phase of the parasite is urgent, especially in rural areas of developing countries where the disease is endemic (Martins et al., 2017; Sheng-Xia et al., 2007; Ke et al., 2017).

Among the various compounds of natural origin that have already been evaluated for their molluscicidal and cercaricidal activity, secondary metabolites such as saponins, terpenes, alkaloids and several phenolic molecules have been highlighted (Lima et al., 2002; Diab et al., 2012; Silva et al., 2009).

Lichens, formed through the symbiotic association between mycobiont and photobiont organisms (algae / cyanobacteria), produce a large variety of secondary metabolites, several of them of phenolic origin, which are produced mainly by the fungus and secreted at the surface of lichens in their amorphous form or in the form of crystals (Mitrović et al., 2011). These substances, usually of low molecular weight, are responsible, in most cases, for the various biological activities conferred to lichens, such as antibiotic (Bellio et al., 2015), antitumor (Delebassée et al., 2017), photoprotective (Dévéhat et al., 2013), analgesic, anti-inflammatory (Bugni et al., 2009), allelopathic (Lokajová et al., 2014), insecticidal (Silva et al., 2009), molluscicidal (Martins et al., 2014) and antiparasitic activities (De Carvalho et al., 2005).

The *Ramalina* genus, belonging to the Ramalinaceae family, comprises a variety of fruticose lichens, with over 200 species registered worldwide (Oh et al., 2014). Several secondary metabolites of biological relevance are produced by species of the genus, such as usnic, sekikaic, diffrataic, salazinic, nortistic, protocetraric and divaricatic acids. This diversity of molecules confers upon species of the genus *Ramalina* bioactivities such as antimicrobial, antioxidant, antiviral, antitumor, cytotoxic, anthelmintic and insecticidal (Moreira et al., 2015), demonstrating the potential of the genus in biological studies.

In this study, the molluscicidal and cytotoxic activities of *Ramalina aspera* on mollusks of the species *Biomphalaria glabrata*, vectors of schistosomiasis mansoni, were evaluated, verifying their effects on the embryonic stages of development and on adult individuals. Concurrently, the activity of the ether extract of *R. aspera* on *Schistosoma mansoni* cercariae and its environmental toxicity were evaluated through the bioassay using the microcrustacean *Artemia salina*.

2. Materials and methods

2.1. Lichen material

Lichens of the species *Ramalina aspera* were collected in the municipality of Limoeiro, state of Pernambuco, Brazil. Species identification was confirmed and a specimen voucher was deposited at the UFP Herbarium, Department of Botany, Federal University of Pernambuco, Brazil (voucher n° 54,299). After collection, the lichen material was kept at room temperature (28 ± 3 °C) until its stalks were completely dry for subsequent preparation of the extract.

2.2. Biomphalaria glabrata mollusks

The geographical lineage comes from São Lourenço da Mata, Pernambuco, Brazil, maintained for successive generations at the Laboratory of Radiobiology of the Department of Biophysics and Radiobiology (UFPE).

2.3. Schistosoma mansoni strain

The strain is maintained at the Laboratory of Immunopathology Keizo Asami (LIKA) of the Federal University of Pernambuco (UFPE), by means of successive passages in snails of the species *Biomphalaria glabrata* kept in the Department of Tropical Medicine (UFPE).

2.4. Preparation of the ether extract

Obtaining the ether extract was performed following the methodology of Asahina and Shibata (1954). Approximately 10 g of the dry lichen sample was subjected to successive extractions with diethyl ether in a soxhlet apparatus at 40 °C. After obtaining the ether extract, the solvent was evaporated in a water bath at a temperature of 40 °C. The dried lichen extract was then conditioned in a desiccator until the time of the experiments.

2.5. High-performance liquid chromatography

In order to determine the lichen metabolites present in the ether extract of *R. aspera*, High-Performance Liquid Chromatography was performed, following the methodology described by Legaz and Vicente (1983), using a Hitachi chromatograph (655 A-11, Tokyo, Japan) coupled to a UV detector at 254 nm. For the separation of the metabolites, a reverse phase C-18 column (MicroPack MCH-18, 300 x 4 mm, Merk® KGaA, Darmstadt, Germany) was used. The mobile phase was composed of methanol / deionized water / acetic acid (80: 19.5: 0.5 v / v / v) in an isocratic system. Other analytical parameters were the following: injection volume 20 mL, attenuation of 0.16, pressure at 87 atm, flow rate of 1.0 mL min^{-1} at room temperature (28 ± 3 °C). Samples were injected at a concentration of 1.0 mg mL^{-1} diluted in Merk® chloroform.

2.6. Bioassays

2.6.1. Toxicity test on different embryonic stages of Biomphalaria glabrata

The evaluation of embryotoxicity was performed following the methodology described by Rapado et al. (2013). To facilitate the collection of *B. glabrata* egg masses from the breeding aquaria (containing from 50 to 60 animals each), 8 × 8-cm polyethylene sheets were used, where the mollusks performed oviposition. Afterward the sheets collected with the egg masses were placed in Petri dishes and the embryos were analyzed and classified according to the embryonic stage using a stereoscopic microscope (Wild M3B, Heerbrugg, Switzerland) and the criteria established by Kawano et al. (1992). Groups of 100 embryos were separated for each embryonic stage: blastula, gastrula, trochophore and veliger phases, and were exposed for 24 h to the ether extract of *R. aspera* at concentrations of 10, 15, 20, 25 and 30 µg mL^{-1}. As negative controls, filtered and dechlorinated water (pH 7.0) (Control 1) and 0.5% DMSO in filtered and dechlorinated water (pH 7.0) (Control 2) were used. As a positive control, a solution of Niclosamide (Bayluscide, Bayer) at a concentration of 1 µg mL^{-1} was used. After exposure, the embryos were washed and replaced in filtered and dechlorinated water (pH 7.0). Embryos were observed for seven days or until the hatching of the control group (Control 1). Embryos at the end of the bioassay were classified as viable and unviable (malformed and dead). The experiment was carried out in triplicate. Thus, in total 300 embryos per concentration for each stage.

2.6.2. Molluscicidal evaluation and effects on the fertility and fecundity of B. glabrata

The molluscicide assay was performed according to World Health Organization guidelines (1965). Adult snails of the species *B. glabrata* (n = 10) aged 2–3 months and having shell diameters of 12–16 cm were selected, verified for sexual maturity by laying egg masses, placed in containers (60 mL) and exposed to the extract (6.5, 7.5, 8.5 and 9.5 µg mL^{-1}) for 24 h (25 ± 3 °C). Subsequently, the snails were washed with filtered and dechlorinated water (pH 7.0), observed for 8 consecutive days and evaluated for mortality. For negative controls 1 and 2 and the positive control, the same specifications of item 2.6.1

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were followed. As classification criteria, the individuals which presented absence of movements, retraction into the shell, the release of hemolymph and absence of heart beats were considered dead. The assay was performed in triplicate.

In order to evaluate the effects of *R. aspera* on fertility and fecundity of *B. glabrata*, eggs laying by snails that survived after being exposed to 6.5, 7.5 and 8.5 μg mL^{-1} of the *R. aspera* extract were recorded for 7 consecutive days starting 48 h after exposure, and analyzed until the complete hatching of the embryos of control group 1. Embryos were counted and classified as viable and unviable (dead and malformed). The experiment was carried out in triplicate.

2.6.2.1. Effects on the morphology and viability of B. glabrata hemocytes. The snails (n = 20) were exposed to sublethal concentrations of the ether extract of *R. aspera* (6.5, 7.5 and 8.5 μg mL^{-1}) for 24 h. After the exposure period, the extract solutions were replaced with filtered and dechlorinated water (pH 7.0) for another 24 h. For negative controls 1 and 2, the same specifications of item 2.6.1 were followed. Of the surviving mollusks, 4 individuals from each group were randomly selected to collect 120 μL of hemolymph, which was subsequently used for morphological analysis and quantitative hemocyte analysis.

The morphological analysis was performed according to the methodology established by Pavlica et al. (2000), with modifications. Samples of 100 μL of hemolymph were deposited on microscopy slides to which 100 μL of 10 mM EDTA (ethylenediaminetetraacetic acid) diluted in Ringer's solution was also added. The slides were conditioned in a humidity chamber for 30 min. After this time the cells were fixed with 200 μL of 1% glutaraldehyde diluted in Ringer's solution for 5 min. The slides were then washed with Ringer's solution and stained with Giemsa (10% in distilled water) for 7 min. Hemocytes (n = 1000) were analyzed in quadruplicate using a binocular optical microscope in a 100× objective. Quantitative cell analysis was performed according to Cajaraville et al. (1996) with modifications. An aliquot of 10 μL of hemolymph was collected, diluted 1:1 in Ringer's solution and applied to a hemocytometer for total cell count, and the following formula was applied to estimate the number of cells per mL of hemolymph:

$$\text{Number of Cells/mL of hemolymph} = \frac{\text{Total number of cells}}{\text{Number of counted quadrants}} \times \text{dilution factor} \times 10{,}000$$

2.6.3. Lethality test on S. mansoni cercariae

B. glabrata snails infected with *S. mansoni* (Belo Horizonte strain) were exposed to artificial light for 2 h to stimulate the release of cercariae used for the bioassay. Following the methodology of Martins et al. (2014), approximately 100 cercariae were exposed to 2 mL of ether extract of *R. aspera* at concentrations of 5, 10, 20 and 30 μg mL^{-1} (28 °C ± 3), which was previously diluted with 0.5% DMSO in filtered and dechlorinated water (pH 7.0). For the negative controls 1 and 2 and the positive control, the same specifications of item 2.6.1 were followed. The analysis was performed at the intervals of 15, 30, 60 and 120 min with a stereoscopic microscope (Wild M3B, Heerbrugg, Switzerland). The assay was performed in triplicate. The results were expressed as follows: 100% of death (+++), higher than 50% of cercariae death (++), less than 50% of cercariae death (+) and absence of death (-).

2.6.4. Bioassay with Artemia salina

The environmental toxicity assessment was performed using the *Artemia salina* bioassay as described by Meyer et al. (1982). Microcrustacean cysts were placed in a beaker with 500 mL of filtered seawater (pH 8.0) and subjected to constant aeration for hatching. After 48 h, the nauplii were placed in test tubes and submitted to concentrations of 25, 50, 75 and 100 μg mL^{-1} (25 °C ± 3) of ether extract of *R. aspera* diluted in 0.5% DMSO in filtered seawater. As negative controls 1 and 2, filtered seawater (Control 1) and 0.5% DMSO in filtered sea water (Control 2) were used. Ten nauplii per concentration and four replicates were used. After 24 h of exposure, the *Artemia* were evaluated for mortality using a stereoscopic microscope (Wild M3B, Heerbrugg, Switzerland).

2.7. Statistical analyses

The results are expressed as mean and standard deviation (SD) and/or percentage (%) for fertility and fecundity analysis of *B. glabrata*, morphological and quantitative hemocytes analysis, lethality test on *S. mansoni* cercariae and bioassay with *Artemia salina*. The estimated lethal concentrations for 10, 50, and 90% of the specimens (LC_{10}, LC_{50}, and LC_{90}) were performed by Probit analysis with 95% confidence interval, for the embryotoxicity test and molluscicidal analysis with *B. glabrata*. The Probit Regression was calculated by the Finney method where the results were obtained by the ratio of Log (dose) and probit values. The variables used to obtain LC were the different concentrations of extracts, the number of non-viable individuals (dead or malformed embryos and dead snails) and the total amount of individuals per concentration. Log transformation was performed automatically by the statistical program StatPlus® 2009 Professional software (AnalystSoft, Canada).

3. Results

3.1. Identification of the components of lichen material

Chromatographic analysis of phenolic metabolites of the ether extract of *Ramalina aspera* showed the presence of 8 peaks in the spectrum, with the presence of divaricatic acid (RT 12.92), reaching 92.51% of the phenolic composition of the extract (peak 6), and other metabolites in low proportions where the maximum percentages observed were 1.16% of peak 7 (RT 18.40) and 1.65% peak 8 (RT 34.04) (Fig. 1). Among the 8 observed peaks in the spectrum, peak number 2 corresponds to the solvent used.

3.2. Bioassays

3.2.1. Biological effects of the ether extract of R. Aspera on B. glabrata

The results of the embryotoxic activity of *R. aspera* on the four embryonic stages of *B. glabrata* and adult *B. glabrata* mollusks with the values of LC_{10}, LC_{50}, and LC_{90} are reported in Table 1. A dose-dependent increase in embryo sensitivity was observed according to the evolution of the embryonic stages, where the blastula and gastrula stages were 100% unviable at a concentration of 30 μg mL^{-1}, while the trochophore and veliger stages reached the same percentage of unviability at the concentration of 20 μg mL^{-1}. For the LC_{90} (95% CL), the values found were 22.78 (22.59–22.97), 24.23 (24.04–24.42), 16.63 (16.39–16.87), and 16.03 (15.76–16.30) μg mL^{-1} for the blastula, gastrula, trochophore and veliger, respectively.

All concentrations tested showed mortality of adult *B. glabrata* mollusks, reaching 100% at the concentration of 9.5 μg mL^{-1}. The remaining concentrations had the survival percentage of 63.3%, 36.6%, and 13.3% for the concentrations of 6.5, 7.5, and 8.5 μg mL^{-1}, respectively. Lethal concentrations (95% CL) were 5.37 (5.18–5.56), 7.0 (6.83–7.21), and 8.66 (8.48–8.86) μg mL^{-1} for CL_{10}, CL_{50}, and CL_{90}, respectively (Table 1).

Fig. 2 shows the changes in *B. glabrata* embryos submitted to the ether extract of *R. aspera*. In addition to mortality, abnormalities such as shell malformations, hydropic embryos, non-specific malformations and delayed embryonic development were observed (Fig. 2 f–g).

The results of the fecundity analysis of *B. glabrata* exposed to the *R. aspera* extract after 7 days of observation are shown in Fig. 3. The data showed a decrease in egg mass deposition in all groups of snails exposed

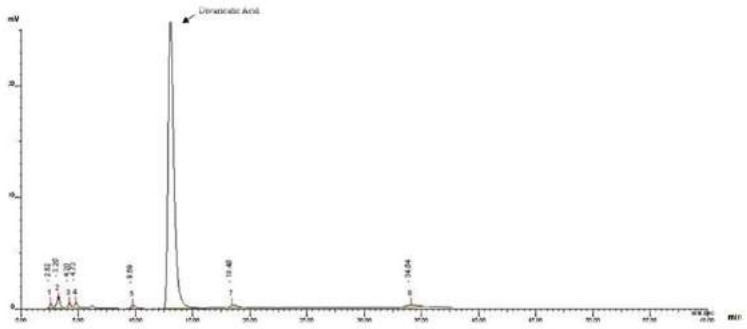

Fig. 1. High-Efficiency Liquid Chromatography of *Ramalina aspera* ether extract.

Table 1
Lethal concentration (LC) for *Biomphalaria glabrata* embryos and adult snails exposed to ether extract of *Ramalina aspera*.

Exposure Time	Concentration (µg/mL)		
	LC_{10} (95% CL)	LC_{50} (95% CL)	LC_{90} (95% CL)
Embryos			
Blastula	11.98 (11.79-12.17)	17.38 (17.19-17.57)	22.78 (22.59-22.97)
Gastrula	13.79 (13.60-13.98)	19.01 (18.82-19.20)	24.23 (24.04-24.42)
Trocophore	8.99 (8,75-9.23)	12.81 (12.57-13.05)	16.63 (16.39-16.87)
Veliger	4.41 (4.13-4.68)	10.55 (10.28-10.82)	16.03 (15.76-16.30)
Adult Snails			
24 hours	5.37 (5.18 – 5.56)	7.0 (6.83 – 7.21)	8.66 (8.48 – 8.86)

Control 1: filtered and dechlorinated water. Control 2: 0.5% DMSO in filtered and dechlorinated water. Niclosamide at a concentration of 1 µg/mL. Significant results were compared with control 2. The significance levels are expressed by * ($p < 0.001$).

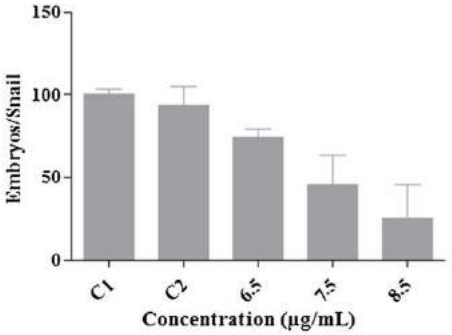

Fig. 3. Fecundity of *B. glabrata* snails exposed to the *R. aspera* extract at the end of the 7-day analysis period. Control 1: filtered and dechlorinated water. Control 2: 0.5% DMSO in filtered and dechlorinated water. The results are expressed as mean and standard deviation (SD).

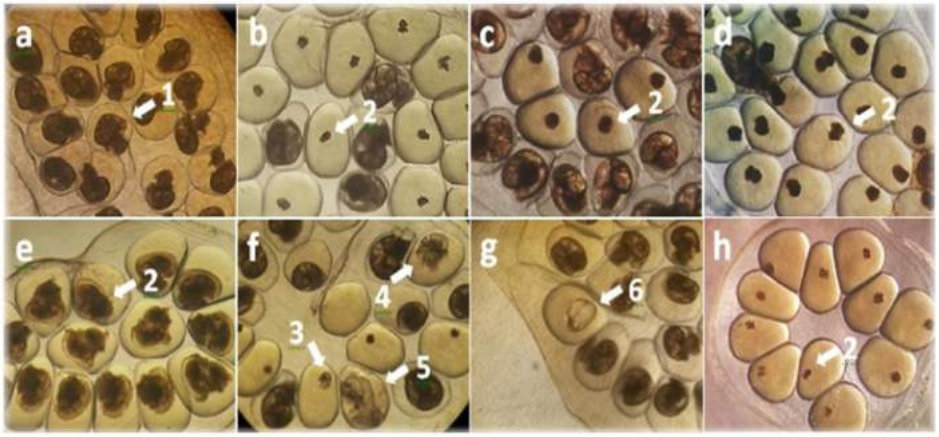

Fig. 2. Embryos of *B. glabrata* in different embryonic stages exposed to the ether extract of *R. aspera* (a–h, x400). **a** - Negative Control **1**. **b** - Blastula stage at 20 µg mL^{-1}. **c** - Gastrula stage at 15 µg mL^{-1}. **d** - Trocophore stage at 15 µg mL^{-1}. **e** - Veliger stage at 10 µg mL^{-1}. **f** and **g** - Blastula stage 15 µg mL^{-1}. **h** - Positive control (niclosamide at 1 µg mL^{-1}). **g** - hydrophobic embryo. **1** - Normal embryo. **2** - Dead embryo. **3** - Non-specific malformation. **4** - Shell malformation and developmental delay. **5** - Shell malformation.

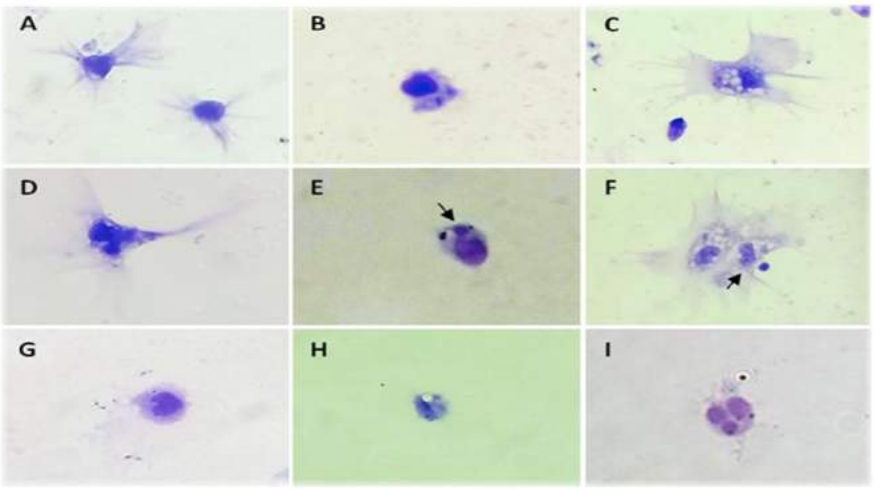

Fig. 4. *B. glabrata* hemocytes after exposure to the ether extract of *R. aspera*. (A) Granulocytic hemocytes. (B) Hyalinocytic hemocyte. (C) Granulocytic hemocyte in apoptosis. (D) Binucleated granulocytic hemocyte. (E) Hyalinocytic hemocyte with micronucleus (F) Granulocytic hemocytes in apoptosis, one of them binucleated (arrow). (G) Hyalinocytic hemocyte with a kidney-shaped nucleus. (H) Hyalinocytic hemocyte with a cytoplasmic vacuole. (I) Trinucleated hyalinocytic hemocyte.

to the ether extract of *R. aspera* when compared to C2. The reduction rates were 20.87%, 51.83% and 73.11% for the concentrations of 6.5, 7.5, and 8.5 μg mL^{-1}, respectively. No differences were observed between the embryonic viability (fertility) of the snail groups exposed to the *R. aspera* extract and the C2 control group.

In the cytomorphological analysis of *B. glabrata*, two hemocyte cell types were observed submitted to *R. aspera* extract. The first cells were of a larger size, star-shaped, presenting adhesions and forming pseudopodia. The second group of cells had a higher nucleus/cytoplasm ratio, homogeneous cytoplasm, with no granules. The extract of *R. aspera* caused morphological changes at all concentrations evaluated. Among the most frequent alterations are binucleations, micronucleations, and apoptotic cells (Fig. 4). Other punctual alterations were also found in fewer numbers, such as trinucleations, kidney-shaped nuclei, and cytoplasmic vacuoles.

The results for the most significant numerical changes, as well as hemocyte count / mL of hemolymph, are described in Fig. 5. Binucleated cells had a frequency and mean ± standard deviation of 0.66% (6.66 ± 0.15), 0.43% (4.33 ± 0.11), and 0.46% (4.66 ± 0.15) at the concentrations of 6.5, 7.5, and 8.5 μg mL^{-1}, respectively. Micronuclei were also found at all concentrations analyzed, where the results of the evaluation revealed 0.23% (2.33 ± 0.11), 0.56% (5.66 ± 0.20) and 0.36% (3.66 ± 0.11) of micronuclei frequency and mean ± standard deviation at the concentrations of 6.5, 7.5, and 8.5 μg mL^{-1}, respectively. Apoptosis hemocytes were observed in higher amounts than the other cellular alterations, presenting percentages and mean ± standard deviation of 3.26% (32.66 ± 0.47), 8.16% (81.66 ± 1.16), and 36.43% (364.33 ± 6.46) at the concentrations of 6.5, 7.5, and 8.5 μg mL^{-1}, respectively. The count of hemocitary cells evidenced an increase in the number of hemocytes as a function of the increase of the concentration to which the mollusks were exposed. However, the values were close to each other, except for the concentration of 8.5 μg mL^{-1}, where there was an increase of 226.38% of hemocitary cells in relation to the negative control.

3.2.2. Cercaricidal activity of the ether extract of R. aspera on S. mansoni cercariae

Table 2 shows the lethal effects of the ether extract of *R. aspera* on *S. mansoni* cercariae at different concentrations. Changes in the motility of cercariae began to be observed at the concentration of 5 μg mL^{-1} in 30 min of exposure. The cercariae presented atypical rotation and vibration movement, moving more slowly with repeated contractions in short intervals of time. Initial mortality results were found at the concentration of 5 μg mL^{-1} in 60 min of exposure (+), reaching 50% mortality (++) at the same concentration in 120 min of exposure. Complete elimination of cercariae (+++) was observed at the concentration of 10 μg mL^{-1} in 120 min of exposure. At all concentrations tested, mortality data were found, with a characteristic of this lichen material being a rupture between the cercarian bodies and tail starting at the concentration of 10 μg mL^{-1}.

3.2.3. Environmental toxicity through the bioassay with A. salina

Among the four concentrations tested, only the two highest, 75 and 100 μg mL^{-1}, presented lethality values. The percentages and mean ± standard deviation of survival of *A. salina* were 100% (0.0 ± 0.0), 100% (0.0 ± 0.0), 90% (9.0 ± 0.81), and 87.5% (8.75 ± 1.25) at the concentrations of 25, 50, 75, and 100 μg mL^{-1}, respectively.

4. Discussion

According to the results obtained, it can be observed that the extract increases its effect with the evolution of embryonic development. In this way, the initial stage of development (blastula) presented 100% lethality in the 30 μg mL^{-1} concentration, decreasing to 20 μg mL^{-1} in the veliger stage. In addition to lethality, *R. aspera* extract caused malformations in the embryos at all concentrations evaluated (Fig. 2). Similar results were found by several authors with lichen materials (Araújo et al., 2018a; Silva et al., 2018). Araújo et al. (2018b) verified the embryotoxic activity of the usnic acid dibenzofuran extracted from lichen *Cladonia substellata* on *B. glabrata*, finding significant results at low concentrations, with LC_{50} of 1.38, 3.47, 5.11 and 2.93 μg mL^{-1} for the blastula, gastrula, trochophore and veliger stages, respectively. In contrast, studies by Martins et al. (2017), using the ether extract of *Cladia aggregata* and the purified barbatic acid of this same lichen, showed that the extract had significant molluscicidal activity, obtaining LC_{50} of 19.9 μg mL^{-1} for the blastula embryonic stage, while the purified compound showed no activity on the embryos. Similarly, the present study confirmed the molluscicidal effect of the ether extract of

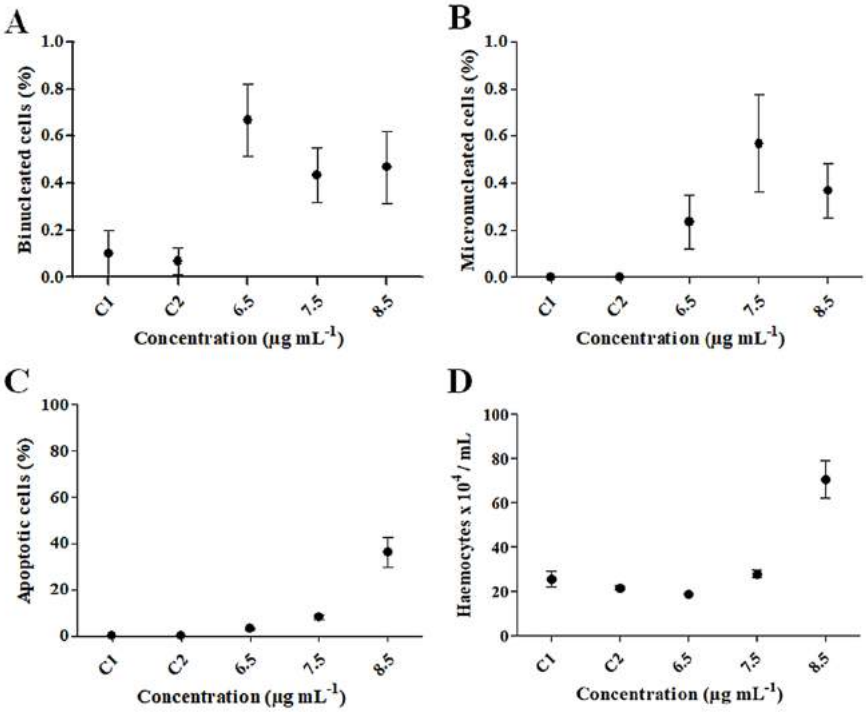

Fig. 5. Morphological and quantitative changes of *B. glabrata* hemocytes exposed to the ether extract of *R. aspera*. **(A)** Binucleated hemocytes. **(B)** Micronucleated hemocytes. **(C)** Apoptotic hemocytes. **(D)** Hemocyte count / mL hemolymph. C1 (Control 1): filtered and dechlorinated water. C2 (Control 2): 0.5% DMSO in filtered and dechlorinated water. The results are expressed as mean and standard deviation (SD).

Table 2
Cercariae mortality after exposure to *R. aspera* extract in relation to exposure time.

Experimental groups (µg mL^{-1})	Exposure time (minutes)			
	15	30	60	120
Control 1	–	–	–	–
Control 2	–	–	–	–
Niclosamide	+++	+++	+++	+++
Ramalina aspera				
5	–	–	+	++
10	–	+	++	+++
20	–	++	+++	+++
30	+	+++	+++	+++

Control 1: filtered and dechlorinated water. Control 2: 0.5% DMSO in filtered and dechlorinated water. Niclosamide at a concentration of 1 µg/mL. Total elimination of cercariae (+++), elimination higher than 50% of cercariae (++), less than 50% elimination of cercariae (+) and absence of lethality (-).

R. aspera on embryos of *B. glabrata* at low concentrations on all the embryonic phases evaluated in a 24-h period (Table 1).

The molluscicidal activity of *R. aspera* was also observed on adult *B. glabrata* mollusks at low concentrations, reaching 100% lethality at the concentration of 9.5 µg mL^{-1}. The ether extract of *R. aspera*, divaricatic acid was the major compound of the extract. A study by Silva et al. (2018) demonstrated that a concentration of 5.5 µg mL^{-1} of divaricatic acid has molluscicidal activity. Therefore, it is suggested that this substance is responsible for the molluscicidal and embryotoxic activity of this ether extract. Studies report the efficacy of various compounds rich in phenols for mollusk control. Upadhyay and Singh (2011) evaluated the molluscicidal activity of *Terminalia chebula* on the snail *Lymnaea acuminata*, a mollusk transmitter of fascioliasis. The biological activity of *T. chebula* was attributed to the presence of tannic acid, a phenolic compound, which was one of the major compounds present in the extract. In the study by Faria et al. (2018), quercetin and myricetin, present in the hydroalcoholic extract of *Manikara subsericea*, proved to be the substances responsible for their molluscicidal activity.

Changes in the reproduction of *B. glabrata* were also confirmed through this study, where it was possible to observe a significant decrease in the reproduction rates of snails exposed to the two highest concentrations (7.5 and 8.5 µg mL^{-1}). As with the *R. aspera* extract, the ether extract of the lichen *Cladia aggregata* also caused a decrease in mollusk reproduction rate and malformations (Martins et al., 2017). In the ether extract of *C. aggregata*, the major compound found was barbatic acid, a depside lichen compound, as well as divaricatic acid, corroborating the similarity in the results found. Lesions in the mollusk reproductive system have been reported after exposure to a natural molluscicidal agent, such as a *Haplophyllum tuberculatum* extract, which caused changes in the sexual hormones of *B. alexandrina*, such as a decrease in egg mass deposition, necrosis in gonad tissues and destruction of oocytes. The authors suggested that the decrease in egg mass deposition caused by *H. tuberculatum* was mainly due to the inhibition of testosterone biosynthesis and decreased levels of progesterone (Rizk et al., 2012).

According to the World Health Organization (WHO, 1983), extracts of vegetable origin are classified as molluscicidal agents when they

exhibit 90% lethality in snails at concentrations equal to or less than 100 μg mL^{-1}. Thus, it is observed that the ether extract of *R. aspera* falls within the parameters established by the WHO both in its activity on embryos of *B. glabrata* and in its activity on adult snails, besides causing a decrease in the reproduction of the snails at the concentrations tested.

The immune system of mollusks consists of cellular and humoral elements, of which hemocytes are the main line of cellular defense of these animals (Ibrahim et al., 2018). According to Galloway and Depledge (2001), the immune system of invertebrates can provide relevant information regarding the chemical compounds to which they are exposed, being of great importance in ecotoxicological studies. The exposure of *Biomphalaria alexandrina* to the aqueous extract of *Anagallis arvensis* at LC_{25} decreased the number of circulating hemocytes and caused alterations such as condensation of the nuclear material on the internal side of the membrane, partial rupture of the cellular organelles, accumulation of vacuoles in the outer membrane and degenerated hyalinocytes with organelles in a difficult state of identification. The same was observed with a *Calendula micranta* extract, which triggered degenerative changes in *B. alexandrina* hemocytes, nuclear vacuolization, and necrosis of cytoplasmic organelles. Again, nuclear condensation was clearly identified (Kamel et al., 2007). Similar results were reported in the study by Mohamed et al. (2006), which describes nuclear and cytoplasmic morphological changes in *B. alexandrina* hemocytes exposed to a *Melia azadrach* extract such as nuclear budding, loss of nuclear membrane definition, chromatin decrease and cytoplasmic vacuoles. However, increased granulocytes were observed as the time of exposure to the extract increased, a result that corroborates the findings of this study. According to Qubella et al. (1993), the variation in hemocyte density may be the result of a reversible migration of tissue cells to the hemolymph and *vice versa*. Another hypothesis for this increase in hemocyte cells would be the mobilization of these cells to the hemolymph for the elimination of apoptotic cells (Russo and Madec, 2007).

A single snail can release tens of thousands of cercariae, the infectious phase of the parasite, which remain viable under suitable aquatic conditions for up to 72 h in search of a definitive host (Colley et al., 2014; Gryssels et al., 2006). In this way, molluscicides that are able to eliminate the vector and the etiological agent of the disease in the same application stand out, providing a more effective combat against parasitosis. In the study conducted by Castro et al. (2015), in which the cercaricidal properties of the ethanolic extract of *Garcinia brasiliensis* were evaluated, it was possible to observe the total elimination of cercariae at concentrations above 12.5 μg mL^{-1} in the period up to 8 h of exposure. El-Beshbishi et al. (2015), when evaluating the efficacy of artemisinin-naphthoquine on *S. mansoni* cercariae, obtained promising results, where the concentration of 16.8 μg mL^{-1} eliminated 100% of the cercariae in 15 min. Like artemisinin-naphthoquine, the ether extract of *R. aspera* also caused a rupture of the cercarian body. The same characteristic has been verified by other authors, corroborating the findings of this study (Castro et al., 2015; Martins et al., 2017; Silva et al., 2018).

The use of a bioassay using *Artemia salina*, a marine microcrustacean, has been considered a useful tool for the preliminary evaluation of toxicity of fungal toxins, plant products, drugs, heavy metals, bacterial toxins, pesticides and pollutants (Carballo et al., 2002; Costa et al., 2015). Martins et al. (2017) evaluated the environmental toxicity of the ether extract of *Cladia aggregata* and barbatic acid depside using a bioassay with *A. salina*, noting that the lichen extract showed no toxicity up to the concentration of 50 μg mL^{-1}, whereas the isolated compound did not present toxicity at any of the concentrations evaluated, with 100% survival at 100 μg mL^{-1}. Similarly, in studies by Silva et al. (2018), divaricatic acid did not prove to be toxic to *A. salina* up to the concentration of 200 μg mL^{-1}.

The results of the present study demonstrate that the ether extract of *R. aspera* can be considered a promising compound for the development of molluscicidal agents that may be used to control the schistosomiasis vector, in both its embryonic stages and its adult phase, as well as in eliminating the etiological agent of the disease in its infectious phase for humans. In addition, the extract showed low environmental toxicity through the *A. salina* bioassay. Therefore, future studies should be considered in order to verify the efficacy of this compound in field tests and using other environmental bioindicators.

Disclosure statement

The authors report no conflicts of interest.

Acknowledgments

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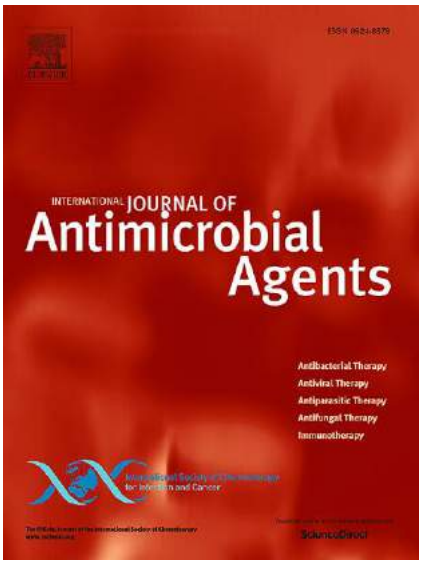
4 ARTIGO 2 - DIVARICATIC ACID FROM *Canoparmelia texana* (LICHEN): *IN VITRO* SCHISTOSOMICIDAL EVALUATION AND ULTRASTRUCTURAL ANALYSIS AGAINST ADULT WORMS OF *Schistosoma mansoni*

ARTIGO SUBMETIDO AO PERIÓDICO



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Divaricatic acid from *Canoparmelia texana* (Lichen): *in vitro* schistosomicidal evaluation and ultrastructural analysis against adult worms of *Schistosoma mansoni*

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Abstract

In this study we evaluated the *in vitro* effect of divaricatic acid from *Canoparmelia texana* against coupled worms of *Schistosoma mansoni*. The schistosomicidal effect was evaluated through the bioassay of motility and mortality, cellular viability of the worms and ultrastructural analysis through Scanning Electron Microscopy. To evaluate the cytotoxicity of divaricatic acid, a cell viability assay was performed with human peripheral blood mononuclear cells. Divaricatic acid proved effect against *S. mansoni* after 3 hours of exposure. At the end of 24 h the concentrations of 100 - 200 μM presented lethality to the worms. Motility changes were observed at sublethal concentrations. The IC_{50} obtained by the cell viability assay for *S. mansoni* was 100.6 μM (96.24 - 105.2 μM). Extensive damage to the worm's tegument was observed such as peeling, erosion, bubbles, edema, damage and loss of tubercles and spines, fissures and tissue ruptures. No cytotoxicity was observed in human peripheral blood mononuclear cells. This report provides data showing the schistosomicidal effect of divaricatic acid on *S. mansoni*, causing death, motile changes and ultrastructural damage to worms. In addition, divaricatic acid was shown to be non-toxic to human peripheral blood mononuclear cells at concentrations effective on S. mansoni.

Keywords: Anthelmintic activity, Divaricatic acid, *Schistosoma mansoni*, Schistosomiasis, Ultrastructural analysis.

1. Introduction

Schistosomiasis, a parasitic infectious disease caused by trematodes of the genus *Schistosoma*, affects around 260 million people worldwide [1], and is considered by the World Health Organization to be the second most devastating disease in terms of public health and socioeconomic impact, after malaria [2, 3]. Prevalent in tropical and subtropical areas, it is considered endemic in 78 countries located in Africa, Asia and Central and South America, mainly affecting the poorest segments of the population who live under inadequate sanitation conditions and without access to clean drinking water [4, 5].

Among the five species of the *Schistosoma* genus of importance to human health (*S. mansoni, S. haematobium, S. japonicum, S. intercalatum* and *S. mekongi*), only *S. mansoni* is the etiologic agent in the Americas, being responsible for the intestinal form of the disease [6]. The main complications resulting from the infection are the result of a granulomatous inflammatory reaction launched in response to egg deposition in the hepatosplenic and gastrointestinal tract of the host, triggering hepatosplenomegaly, portal hypertension, ascites, hematemesis of esophageal varices and fibrosis [5, 7, 8].

Currently, praziquantel (PZQ) is the only drug of choice for the treatment and control of schistosomiasis [7]. Although PZQ is an effective drug, biosecure and is of low cost, the fact that there are no alternative drugs for an expanding disease which affects millions of people around the world is a matter of concern [9]. In addition, PZQ does not treat recent infections, has low efficacy on young worms, does not prevent reinfection and its mass administration, aiming at the eradication of the disease in endemic countries, has led to a decrease in drug efficacy, which portends the selection of strains resistant and/or tolerant to PZQ [7, 10, 11].

The search for new schistosomicidal agents has turned to natural products as a promising source for the appearance of new bioactive molecules [12]. In addition, several population groups make use of these natural products as important tools in traditional local medicine, which facilitates their access and lowers the cost in their acquisition [13].

Lichens are symbiotic associations between photobionts and mycobionts [14]. With a wide distribution across terrestrial habitats, these organisms produce a great diversity of metabolites, in their great majority of phenolic origin, known as lichen substances [15, 16], which aim not only to preserve the symbiotic structure, but also to protect against pathogens

and predators, intense UV radiation and oxidative stress [17]. Among the various classes of metabolites produced by lichens, such as depsidones, dibenzofurans, xanthones, anthraquinones and benzyl ethers, the depsides are distinguished by their diverse biological activities, including antiproliferative [18], antioxidant [19], enzymatic inhibition [20] and molluscicidal and cercaricidal [21] activities. Divaricatic acid belongs to the depside class and is commonly found in lichens of the genus *Canoparmelia* and *Ramalina*; yet it is still rarely mentioned in the literature and its biological potential has remained little known.

In the present study, we evaluated the *in vitro* schistosomicidal potential of divaricatic acid, a lichen substance extracted from *Canoparmelia texana*, on adult worms of *Schistosoma mansoni* through parameters such as motility and mortality analysis, cell viability of worms, evaluation of its effects on morphology and its cytotoxic effect on human peripheral blood mononuclear cells (PBMC).

2. Materials and methods

2.1 Samples of C.texana

Canoparmelia texana lichen was collected in the South region of Brazil, in the city of Curitiba (State of Paraná, Brazil), and kept at room temperature (28 ± 3 ºC), stored in a desiccator for later identification. The identification of the lichen thallus was performed and an exsiccate was deposited in the UFP Herbarium, Department of Botany of the Federal University of Pernambuco (voucher nº 44627).

2.2 Extract of C. texana, *purification and isolation of divaricatic acid*

The lichen sample (approximately 50 g) was subjected to successive extractions with diethyl ether in a Soxhlet apparatus at 30 °C and the solvent was subsequently evaporated at 40 °C. The extract was then dissolved in methanol and crystallized at room temperature. For seven consecutive days, the crystals were withdrawn from the crystallization vials and stored. The crystals obtained were then subjected to successive washing with chloroform in a G4 funnel under pressure. The liquid acquired after washing was conditioned in glass vials and

kept under refrigeration (4 ± 2 °C) for crystallization of divaricatic acid; subsequent chemical analysis were performed to confirm the isolation of the molecule.

2.3 Chemical analysis

2.3.1 Thin-Layer (TLC) Chromatographs and High-Performance Liquid Chromatography (HPLC)

For one dimensional TLC, the methodology of Culberson [22] was followed using a silica gel 60 plate (Merk® PF254 + 366). For elution, the system used was toluene/dioxane/acetic acid (45:12.5:2 - v/v/v) and the development was sprayed with 10% sulfuric acid followed by heating at 50 °C for 5 min. Bands were observed using UV radiation (254 and 366 nm). Samples that presented only one band in TLC were then analyzed by HPLC to verify the percent purity of the sample (> 95%), following the methodology described by Legaz and Vicente [23]. A Hitachi Chromatograph (655 A-11, Tokyo, Japan) coupled to a CG437 UV detector set at 254 nm was used. For the separation process, a C-18 reversed-phase column (MicroPack MCH-18), measuring 300 × 4 mm (Merck® KGaA, Darmstadt, Germany) was used. The samples were injected at the concentration of 1.0 mg/mL diluted in ether (Merk®). The mobile phase was composed of methanol/water/acetic acid (80:19.5:0.5 v/v/v) in an isocratic system. Other analytical parameters were the following: injection volume of 20 mL, attenuation of 0.16, pressure at 87 atm and flow rate of 1.0 mL min^{-1} at room temperature (28 ± 3 ºC).

2.3.2 ^{1}H (^{1}H NMR) and ^{13}C (^{13}C NMR) Nuclear Magnetic Resonance and Infrared (IR) Analysis

For the determination of the structure of the isolated molecule, ^{1}H NMR and ^{13}C NMR were performed using a Varian Unity Plus spectrometer at 400 MHZ, 27 °C, with DMSO-D6 as the solvent. Infrared analysis was performed using a Bruker spectrophotometer coupled to a Fourier transformer (IF model 566) using KBr pellets. The analysis were carried out in the Fundamental Chemistry Department of the Federal University of Pernambuco - UFPE, Brazil.

2.4 Bioassays

2.4.1 Ethical considerations, animals and infection by S. mansoni

To obtain the worms, female *Swiss Webster* mice were used, kept at the Keizo Asami Immunopathology Laboratory - LIKA (UFPE), according to standard conditions of temperature (23 ± 2 ºC), photoperiod (12 h of light/dark), humidity (40-50%) and water and feed (LABINA®) *ad libitum*. *S. mansoni* strain Belo Horizonte (BH), maintained through successive passages in snails of the species *Biomphalaria glabrata* at the Laboratory of Immunology of Parasitic Diseases and Experimental Schistosomiasis - LIKA (UFPE), was used to infect the mice. The mice were infected by percutaneous exposure to 120 cercariae, under intramuscular anesthesia with ketamine and xylazine at a ratio of 2:1 and a dose of 4 mg kg^{-1} [24]. *In vitro* experiments were approved by the Animal Experimentation Ethics Committee of the Biosciences Center, Federal University of Pernambuco (Protocol 23076.036388 / 2014-11).

2.4.2 In vitro studies with S. mansoni

Forty-five days after infection, the mice were euthanized by cervical dislocation and, later, the hepatic portal system and mesenteric vessels were perfused to obtain the worms. Worms were placed in Petri dishes with RPMI 1640 culture medium supplemented with 20 mM HEPES, 100 µg/mL penicillin, 100 µg streptomycin and 10% fetal bovine serum and washed with this medium three consecutive times. Two pairs of coupled worms were distributed per well in 24-well sterile culture plates containing 2mL of the same supplemented medium. The worms were incubated for adaptation in a humid atmosphere containing 5% CO_2 at 37 °C for 2 h. After incubation, divaricatic acid was added at the concentrations of 200, 100, 50 and 25 µM. For the negative controls, we used only RPMI medium and RPMI added 1.5% DMSO. In the positive control, the worms were incubated in 10 µM of PZQ. All experiments were performed in sextuplicate with a minimum repetition of two times (16 pairs of worms per concentration).

2.4.3 Schistosomicidal evaluation criteria

2.4.3.1 Motility and survival

An inverted microscope (Leica Microsystems, DM IL Wetzlar, Germany) was used to evaluate the motility and survival of coupled worms monitored at 3, 6, 12 and 24 h of exposure. Motility and survival of the worms were evaluated according to the following criteria and scored on a viability scale of 3-0: 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 suckers not adhered to the plate walls; 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 [25].

2.4.3.2 Cell viability assay of coupled S. mansoni

In the evaluation of the cytotoxicity of divaricatic acid on worms, the MTT (-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) methodology described by Aires et al. [7] was used. After exposure to divaricatic acid (200, 100, 50 and 25 μM) for 24 h, two worms per well were placed in 96-well plates containing 100 μL MTT (5 μg / mL concentration, diluted in phosphate buffer, PBS) and incubated for 30 min. in a darkroom at 37 °C. Subsequently, the MTT was carefully removed and 200 μl of DMSO was added per well of the plate. The samples were kept under stirring for 1 h at room temperature. This method allows the visualization of mitochondrial enzymatic activity through the formation of purple coloration. Optical densities of the samples were measured at 550 nm in an ELISA microplate reader (M680, Bio-Rad Laboratories, Inc.). The worms of the positive and negative control groups were submitted to the same test. All experiments were carried out in quadruplicate and repeated at least twice. The significant differences were taken at $p < 0.05$.

2.4.3.3 Scanning Electron Microscopy (SEM)

The worms were analyzed by SEM after 24 hours of incubation in divaricatic acid. Worms were fixed in 2.5% glutaraldehyde and 4% 0.1 M sodium cacodylate buffer (pH 7.2) for 12 h at room temperature. After fixation, the samples were washed with this same buffer and post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer for 1 h out of the

light. Subsequently, the samples were washed with 0.1 M phosphate buffer, dehydrated with increasing concentrations of ethanol at 30, 50, 70, 90 and 100% for 10 min. and dried at critical point (Hitachi HCP-2, Hitachi, Japan), using CO_2 as a transitional medium. The samples were mounted on small aluminum supports, metallized with gold on a FINE-COAT 1100-JEOL metallizer (Ion Sputter JFC-1100), for 6 min. The specimens were then examined and photographed with a JEOL-JSM-5600 LV Scanning Electron Microscope (Tokyo, Japan).

2.4.4 Ethical considerations and Cytotoxicity Assay

PBMCs (peripheral blood mononuclear cell), collected from healthy volunteers (n = 5), were used to verify the toxic effects of divaricatic acid on human cells, according to the methodology of Albuquerque et al. [26]. The culture was performed in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/mL streptomycin at 37 °C with 5% CO_2. The method of evaluating cytotoxicity was by MTT, where the cells were placed in a 96-well plate (1 x 10^6 cells / mL) in RPMI 1640 medium and incubated for 24h (37 °C and 5% CO_2). Divaricatic acid was added at an initial concentration of 200 μM and serial dilutions were performed up to the minimum concentration of 1.56 μM, and the plate was incubated for 72 h. For the negative control, cells were used without treatment with divaricatic acid. Three hours before the final incubation time, 25 μL of MTT (5 mg/mL in PBS buffer) was added to each well. After 3 h of incubation, the medium was aspirated and 100 μL of DMSO was added to each well, and the optical density was then measured at 450 nm in ELISA microplate reader (M680, Bio-Rad Laboratories, Inc.). The assays were performed in quadruplicate in 3 independent assays. All donors of blood samples signed an informed consent form and the study was approved by National Health Council Resolution 466/12 (CAAE) 62919816.2.0000.5208.

2.4.5 Statistical Analysis

Significant differences were identified using ANOVA followed by the Student-Newman-Keuls post-test, where $p < 0.05$ was considered significant for all analysis. Statistical analysis were performed using GraphPad Prism 5.0 software for Windows (GraphPad Software, San Diego, CA, USA). The estimated lethal concentration for 50% of the specimens (IC_{50}) was performed by Probit analysis with a 95% confidence interval, using the StatPlus® 2009 Professional software (AnalystSoft, Canada).

3. Results

3.1 Chemical analyses

The TLC of lichen crystals showed a single band, a result compatible with the standard used, presenting an Rf of 0.38, similar to the findings by Marijana et al. [27]. HPLC, performed to confirm the purity of the lichen crystals, showed an Rt of 16.04, compatible with the standard used, and 98.45% purity. ^{1}H and ^{13}C Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR) and Infrared (IR) analysis (**Table 1**) confirmed the molecular structure of divaricatic acid ($C_{21}H_{24}O_{7}$) (**Fig. 1**).

3.2 Changes in cellular motility and viability of coupled worms of S. mansoni *induced by divaricatic acid*

Table 2 shows the motility scores of adult *S. mansoni* worms after exposure to divaricatic acid in the 3, 6, 12 and 24 hour intervals at different concentrations. At all observation intervals, worms from the negative control groups, incubated only in RPMI and RPMI medium with 1.5% DMSO, showed no difference in motility and cell viability. These worms showed typical movements, exhibiting peristalsis of the internal organs, suckers in movement, adhering to the bottom or sides of the culture plate; score 3. Thus, we chose to express the cell motility and viability results of worms incubated in RPMI with 1.5% DMSO.

From 3 hours of exposure it was possible to observe mortality of worms, Score 0 (complete absence of motions and tegument with or without changes in coloration), of 18.75% at the concentration of 200 μM. At this same concentration, 81.25% of worms showed movements only at the extremities or at only one of the extremities (anterior or posterior regions), with absence of peristalsis of the internal organs and suckers not adhered to the plate walls (Score 1). After 6 hours of incubation at a concentration of 200 μM, 37.5% were Score 0 and 62.5% Score 1. At the same time of exposure, 93.75% of the worms exposed to the concentration of 100 μM of divaricatic acid showed reduced movements throughout the body, with the presence of peristalsis of internal organs and movement of suckers (Score 2) and the others were Score 3. At 12 hours of incubation, motility changes began to be observed at the concentration of 50 μM, where 43.75% of the worms were Score 2. At the concentration of 100 μM, 100% of the worms were found in Score 1 after 12 h of

incubation. At the same time of incubation and at the highest concentration evaluated, 200 μM, 87.5% were Score 0, and the others Score 1. At the final time of incubation in divaricatic acid, 24 h, it was possible to observe mortality percentages of 100% (Score 0) at the concentration of 200 μM and of 87.5% at the concentration of 100 μM. At the 50 μM concentration, 62.5% were Score 1 and the others Score 2. The lowest concentration, 25 μM, caused motile changes in 25% of worms (Score 2). In the positive control group, PZQ, changes began to be observed at 3 h of exposure, with mortality (Score 0) at 6 h of exposure in 6.25% of worms. At 24 h, PZQ presented 87.5% of worms Score 0 and 12.5% Score 1.

Cell viability analysis of *S. mansoni* demonstrated that divaricatic acid caused a significant ($p < 0.05$) reduction in the percentage of viable cells at all concentrations as compared to the negative control group. As shown in **Fig. 2**, there was a reduction of 17.05%, 29.55%, 60.23% and 85.23% for the concentrations of 25, 50, 100 and 200 μM, respectively. The IC_{50} (95% CI) obtained was 100.6 μM (96.24 - 105.2 μM). PZQ caused 70.51% reduction in viability of *S. mansoni*.

3.3 Ultrastructural alterations of coupled worms of S. mansoni *induced by divaricatic acid*

In the ultrastructural analysis of the worms through SEM, it was possible to verify that the negative control group (**Fig. 3**), after incubation for 24 h, exhibited ultrastructure and body topography within the normal morphological parameters. **Fig. 3a-b** show the lateral view of a couple of *S. mansoni* worms, where the female (fw) is inserted into the gynecophore canal of the male (mw), showing its attachment structures, ventral (vs) and oral suckers (os). In **Fig. 3c-d**, the ventral region is shown in its anterior portion, and it is possible to observe in detail the suckers of the male worm (**Fig. 3c**) and female (**Fig. 3d**). In **Figs. 3e-f**, the dorsal region, presenting preserved morphology of a male worm (**Fig. 3e**) and female (**Fig. 3f**) are displayed. In the male worm's tegument, a large number of tubercles (tb), dome-shaped papillae (dp) and parallel folds of the tegument (paf) can be seen. In the female worm's tegument, the presence of numerous parallel transverse folds (paf) is visualized. In the *S. mansoni* groups incubated with divaricatic acid (25, 50, 100 and 200 μM), all presented tegumentary changes after 24 h of exposure. The alterations caused by exposure to divaricatic acid begin at 25μM (**Fig. 4a-d**) with the presence of eroded tubercles (TD) with exposure of subtegumentary layer (stl) tissue in male worms (**Fig. 4b-c**) and appearance of small bubbles (B) (**Fig. 4d**). The damages are progressive with the increase of the concentration, being

observed at the 50 μM concentration (**Fig. 4e-h**) a larger amount of bubbles (B) disseminated over the tegument, damage to the tubercles (TD) with the loss of spines (SL), the presence of small holes (HL), projections (P) or large bubbles (B) in its apical region (**Fig. 4f and 4h**). Regions of the tegument with edema (SW), peeling (PL) and erosion (TE) could also be visualized **(Fig 4g)**. **Fig. 5 (a-f)** shows the morphological changes caused by divaricatic acid at the concentration of 100 μM, where the morphological changes in the female worms began. Regions with the presence of bubbles (B) and intense edema (SW) could be visualized in several points of the body of the female and male parasites, being also frequent the loss of tubercular spines (TB) and damage to the tubercles (TD) with exposure of the subtegumentar layer (**Fig. 5b-d**). Regions of retraction of the tegument (TR) and peeling (PL) could also be observed (**Fig. 5f**). The intense contortion of female worms that were uncoupled was a frequent feature at this concentration (**Fig. 5e**).

At the 200 μM concentration (**Fig. 6a–f**) the most critical alterations due to exposure to divaricatic acid were observed throughout the body of the parasite. At this concentration the tegumentary damages in female worms were also present and intensified, as compared to the concentration of 100 μM. Extensive damage throughout the gynecophore (gc) canal, with the presence of tegumentary erosion (TE) and peeling (PL) could be visualized (**Fig. 6 b-c**). As in previous concentrations, loss (TL) and damage (TD) to tubercles with the presence of small bubbles (B), erosion (TE), loss of tubercular spines (SL), swelling (SW) were observed (**Fig. c-e**). At this concentration, the onset of cracks (FS) (**Fig. 6b,e,f**) and damage to the tegument at the muscular level (**Fig. 6f**) of the worms could be visualized.

The positive control group (**Fig. 7a–d**), after exposure to PZQ, exhibited marked muscle contraction, contortion and shortening of worms (**Fig. 7a**). A large number of bubbles was observed in the tegument of the worms, distributed in a diffuse form throughout the body of the parasite. Regions of the tegument presented the formation of small grooves (GV) (**Fig. 7b**), loss and damage of tubercles and peeling (**Fig. 7c-d**).

3.4 Divaricatic acid does not present cytotoxicity against PBMC

No significant levels of cytotoxicity to human PBMC were detected among the concentrations tested (1.56 μM – 200 μM) when compared to the control group. It was not possible to define an IC_{50} (IC_{50} > 200 μM) for the concentrations evaluated.

4. Discussion

The development of new schistosomiasis drugs is of extreme importance given the relevance of schistosomiasis in terms of public health, the absence of an effective vaccine for the control of the disease, and the possibility of the emergence of parasite strains resistant to PZQ, the only available drug for disease control [28]. Within this context, it is observed that a variety of studies have been conducted with the focus on bioactive compounds obtained from natural sources. Compounds of the classes of terpenes, alkaloids, quinones, phenols, flavonoids, neolignans and peptides have already had their schistosomicidal effects proven in *in vitro* and *in vivo* evaluations [12].

Depside also found in lichen species as *Lecanora frustulosa*, *Ramalina aspera* and *Dirinaria aspera* [16, 27, 29], divaricatic acid has already had its biological potential reported in some studies that have demonstrated its antimicrobial, antitumor, molluscicidal and enzymatic inhibition activity [16, 27, 30, 31]. In the present study, divaricatic acid extracted from *Canoparmelia texana* showed dose-dependent schistosomicidal activity *in vitro* against *S. mansoni*, causing changes in motility, reduction of cell viability of worms, tegumentary damage and death. According to our results, divaricatic acid caused 100% and 87.5% mortality in *S. mansoni* after 24 h of incubation at concentrations of 200 and 100 μM, respectively.

In a study carried out by Silva et al. [16], the cercaricidal activity of divaricatic acid was evaluated on *S. mansoni* cercariae. The results obtained confirmed its efficacy on the infective larval stage of the parasite, causing mortality from the concentration of 0.5 μg/mL after 60 min of exposure. From the concentration of 10 μg/mL, the cercaricidal activity, with 100% mortality, was observed after 30 min of exposure. Other compounds of phenolic nature have already demonstrated the promising schistosomicidal potential of this chemical group, as evidenced by the work developed by Magalhães et al. [32], which evaluated the schistosomicidal effect of curcumin, a major metabolite of *Curcuma longa* (Zingiberaceae) rhizome, on *S. mansoni* adult worms, observing percentages of 100% mortality at concentrations of 50 and 100 μM, as well as motility changes at all concentrations evaluated, in a period of 24 hours. However, no tegumentary changes were observed in worms exposed to curcumin at any of the concentrations evaluated. Lorsuwannaratet al. [33], who evaluated plumbagin's schistosomicidal effect on *S. mansoni*, also observed changes in motility and the presence of mortality at all concentrations evaluated over a 24 hour period. Changes in the

tegument were also reported by the authors, similar to those found with exposure to divaricatic acid, such as desquamation, regions with swelling, tubers damaged or with loss of spines and formation and rupture of bubbles. Similarly, Araújo et al. [34] evaluated the schistosomicidal activity of potassium usnate, salt derived from usnic acid extracted from lichen *Cladonia substellata*, and observed progressive damages to the *S. mansoni* tegument, describing the appearance of bubbles, edema, muscle contraction, erosion and disintegration of the tubercles and tegument worms. Another important characteristic of the action of divaricatic acid on the tegument of *S. mansoni* is its performance on female worms. Previous studies have shown that soft tissue changes in male worms are more pronounced than those seen in female worm [35]. Even so, divaricatic acid was able to cause significant tegumentary changes in females at concentrations of 100 and 200 μM.

Although changes in the superficial ultrastructure of *Schistosoma* worms receive little attention, studies with this focus have intensified to evaluate the schistosomicidal effect of various drugs [36]. The external surface of *S. mansoni* adult worms is composed of a syncytium (the tegument) covered by a heptalaminate membrane, a structure unique to the trematodes [9, 37]. Because it is a structure that ensures the functioning of several vital functions, such as nutrient absorption, lipid and cholesterol metabolism, tissue proliferation and repair, and selective absorption of drugs, the *S. mansoni* tegument turns out to be an important target for the action of schistosomicidal drugs [7].

The mechanism of action of divaricatic acid is still not well understood, however studies carried out by Bellio et al. [38] have demonstrated that divaricatic acid, like other depsides, has enzymatic inhibitory activity on an ATPase that plays a crucial role in DNA repair. Several papers report drugs with schistosomicidal potential that interact with important ATPases and ATPDases of *S. mansoni*, such as Artemether, Thapsigargin, Cyclopiazonic acid, ouabain and alkylaminoalkanethiosulfuric acids [39, 40, 41, 42]. One hypothesis for the action of divaricatic acid on *S. mansoni*, besides the severe damage to the worm's tegument, would be its action on ATPases important for its physiology. Considering the results obtained, further studies should be conducted to elucidate the mechanism of action of divaricatic acid on *S. mansoni*.

There are still few studies involving evaluations of divaricatic acid cytotoxicity on non-tumor cells. This is the first report of the cytotoxic effect of divaricatic acid on Peripheral Blood Mononuclear Cells (PBMC’s), non-cancerous human cells. In the present study,

divaricatic acid did not demonstrate significant levels of toxicity at any of the concentrations evaluated, which is a promising result, demonstrating the specificity of divaricatic acid on *S. mansoni* when confronted with this cell line. In a study carried out by Russo et al. [31], the cytotoxic effect of three depsides (atranorin and diffractaic and divaricatic acids) on normal human non-immortalized buccal fibroblast cells (BFC) was evaluated through the MTT assay. At the concentrations evaluated (25 and 50 μM), no significant toxicity levels were observed for any of the substances evaluated.

In conclusion, this is a preliminary evaluation of the schistosomicidal potential of divaricatic acid against *S. mansoni* and non-cancerous human cells. In all bioassays, divaricatic acid proved to be effective in eliminating the parasite, also causing significant ultrastructural damage, within concentrations not toxic to human PMBCs. This study presents significant results, indicating the chemical group of depsides, a class still under study, as possible antiparasitic agents.

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Table 1.^{1}H and ^{13}C NMR and IR analysis of divaricatic acid.

Infrared (KBr) approximate vibrational assignments		^{1}H (400 MH_Z, DMSO-d6) NMR spectroscopic data		^{13}C (400 MH_Z, DMSO-d6) NMR spectroscopic data	
Position	ν (cm^{-1})	Position	δ_H (*J* in H_Z) ppm	Position	δ_C (in ppm)
3''	2994 ν_s (CH_3)	1''	2.59 T (8.0)	1	106.86 (C)
3'''	2872 ν_{as} (CH_3)	1'''	2.63 T (8.0)	2	156.90 (C)
7	1678 ν (C=O) phenyl ester conjugated	2''	1.51 – 1.61 m	3	99.08 (C)
7'	1646 ν (C=O) carboxylic acid	2'''	1.51 – 1.61 m	4	151.72 (C)
1and 2*	1609 ν (C=C) aromatic	3''	0.86 T (8.1)	5	113.15 (C)
	1540				
3''	1328 δ_{as} (CH_3)	3'''	0.91 T (8.1)	6	142.70 (C)
3'''	1284δ_s (CH_3)	4	3.74 s (OCH_3)	7	166.98 (C)
7**	1236 ν (C-O) chain ester	3'	6.35 d (2.2)	1'	111.79 (C)
	1135				
7'	1202 ν (C-O) carboxylic acid	5'	6.36 d (2.2)	2'	157.93 (C)
		3	6.50 d (2.4)	3'	106.98 (C)
		5	6.58 d (2.4)	4'	161.73 (C)
		2	10.26 s (OH)	5'	118.88 (C)
		2'	10.26 s (OH)	6'	143.44 (C)
				7'	169.78 (C)
				1''	35.48 (C)
				2''	24.22 (C)
				3''	13.87 (C)
				4''	55.21 (C)
				1'''	35.78 (C)
				2'''	24.94 (C)
				3'''	13.91 (C)

* Positions 1 and 2 correspond to the two absorptions of the double bond of the aromatic ring.
** Position 7 corresponds to the two absorptions of the ester carbonyl.

Table 2. Motility scores of adult *S. mansoni* worms treated with divaricatic acid at different concentrations and exposure times.

Groups	Percent of worms (%) in motility scores after incubation															
	3h				6h				12h				24h			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Controls				100				100				100				100
PZQ																
10 µM		81.25	18.75		6.25	93.75			62.5	37.5			87.5	12.5		
Divaricatic Acid																
200 µM	18.75	81.25			37.5	62.5			87.5	12.5			100			
100 µM			31.25	68.75			93.75	6.25		100			87.5	12.5		
50 µM				100			6.25	93.75			43.75	56.25		62.5	37.5	
25 µM				100				100				100			25	75

Note: percentage values of 32 worms (16 pairs of worms per concentration) per group.
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 and suckers. Slow peristalsis of the organs.
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 suckers not adhered to the plate walls.
Score 0 = complete absence of motion and tegument with or without changes in coloration.

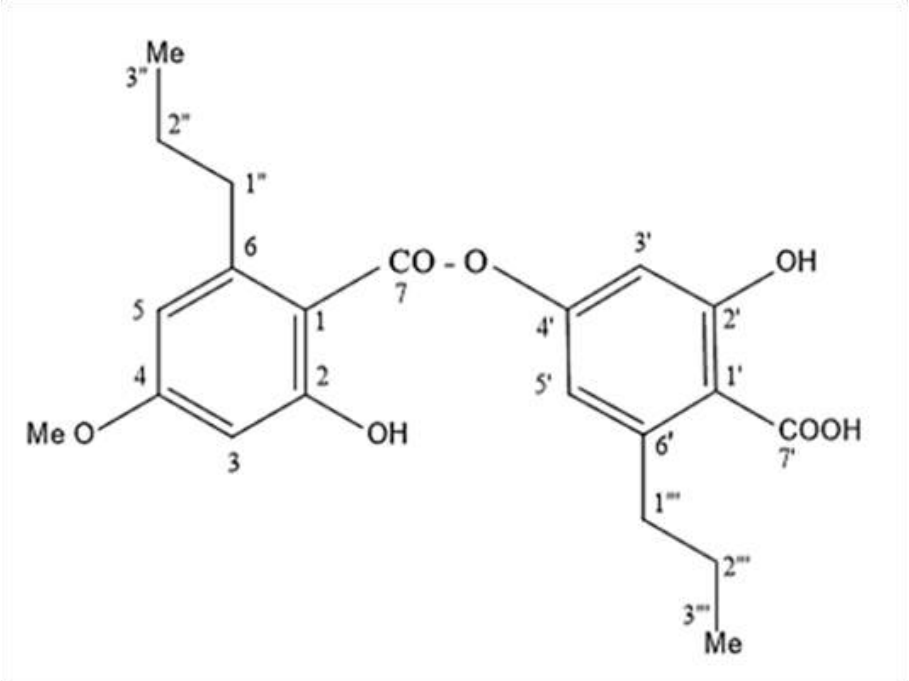


Fig. 1. Molecular structure of divaricatic acid

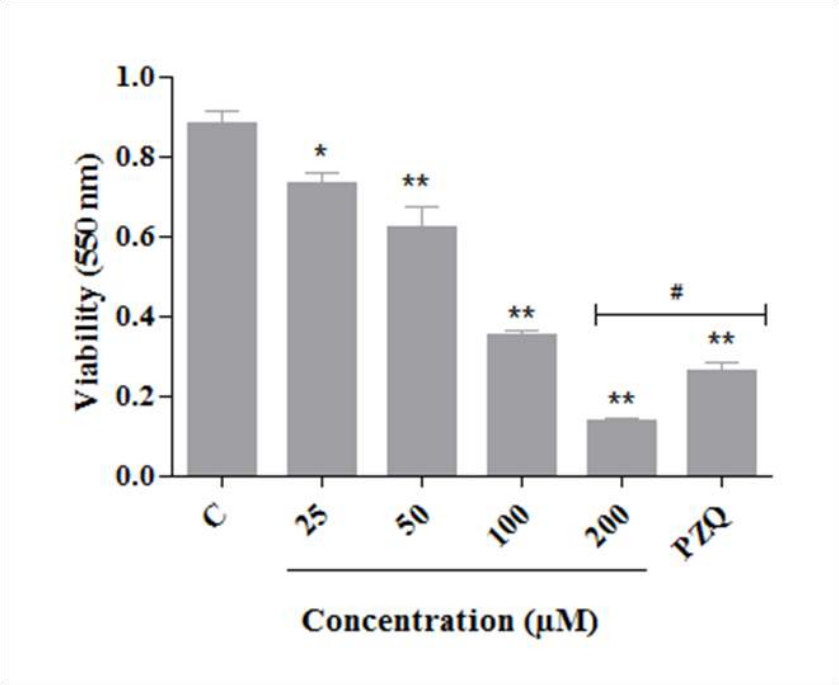


Fig. 2. *In vitro* effects of divaricatic acid (25, 50, 100 and 200 µM) on the cell viability of coupled *S. mansoni* worms after 24 h incubation by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) methodology. Viability was expressed as the mean ± SD of the absorbance values. C, negative control group. * $p < 0.05$ when compared to C. ** $p < 0.001$ when compared to C. # $p < 0.001$ when compared to the positive control (PZQ).

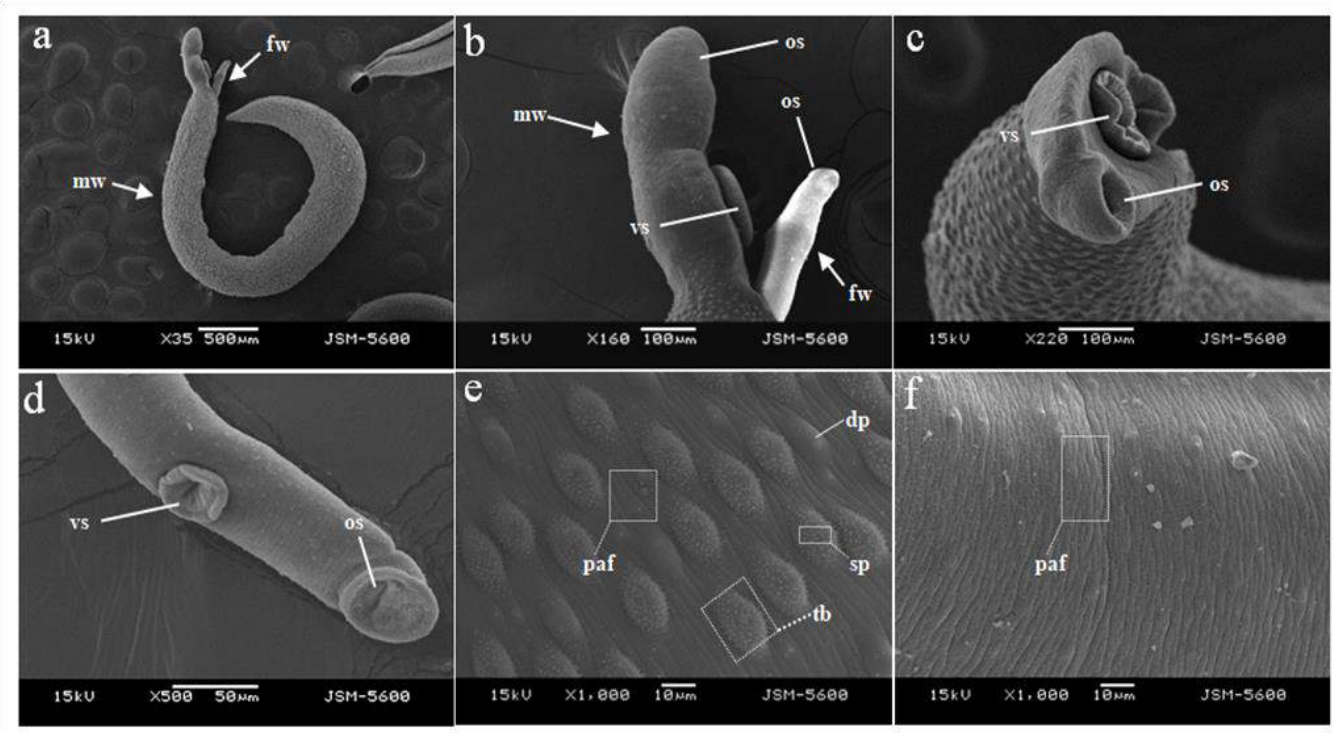


Fig 3. **(a-f)** SEM images of male and female worms of *S. mansoni* from the Negative Control group (1.5% DMSO) after incubation for 24 h. (**a**, x35) Lateral curvature of male (mw) and female (fw) of mated *S. mansoni*. (**b**, x160) Approximate image of **a**, where the male (mw) exhibits oral (os) and ventral (vs) suckers and female (fw) displays the oral sucker (os). (**c**, x220) Anterior region of male worm, detailing the well-preserved morphology of its suckers (os and vs). (**d**, x500) Anterior region of female worm, exhibiting oral (os) and ventral sucker (vs) in detail. (**e**, x1000) Dorsal region of male worm showing tubercles (tb), spines (sp), dome-shaped papillae (dp) and parallel transverse folds of the tegument (paf) without abnormalities. (**f**, x1000) Approximate image of the female worm's tegument showing the presence of parallel transverse folds (paf).

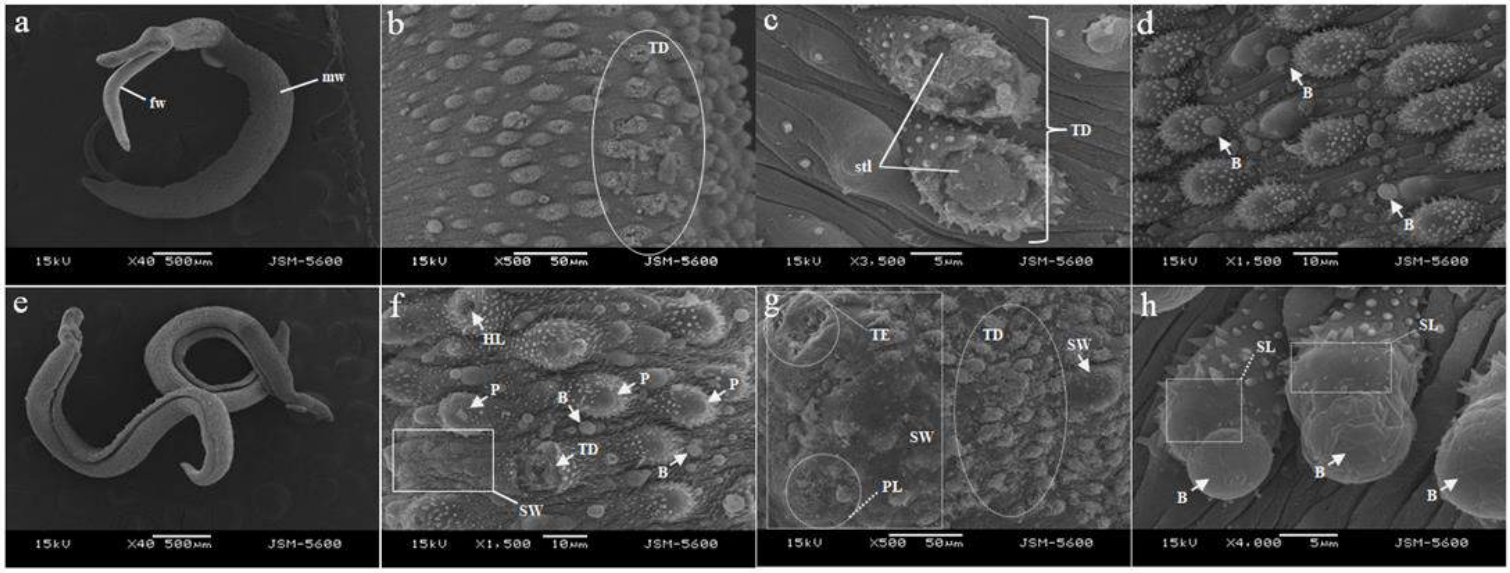


Fig. 4. (**a – h**) SEM images of adult *S. mansoni* worms exposed for 24 h at concentrations of 25 µM **(a – d)** and 50 µM (**e – h**) of divaricatic acid. (**a**, x40) Worm couple, where the male (mw) exhibits dorsal curvature with female (fw) housed in its gynecophore canal. (**b**, x500) Dorsal region of a male worm with focal tubercular damage (TD). (**c**, x3500) Tubercles damaged (TD) with exposure of the subtegumentar layer (stl). (**d**, x1000) Tegument of the male worm presenting several bubbles (B). (**e**, x40) Two male worms exhibiting ventral and dorsoventral curvature. (**f**, x1500) Tegument presenting small bubbles (B), projections of the integument (P), emergence of tubercular orifices (HL), tubers damaged (TD) and eroded with exposure of the subtegumentar layer and regions of edema (SW). (**g**, x500) Tegument of male worm with presence of damage of tubercles (TD), tegumentary erosion (TE), peeling (PL) and edema (SW). (**h**, x4000) Approximate image of tubercles with bubbles (B) on their surfaces and regions with loss of spines (SL).

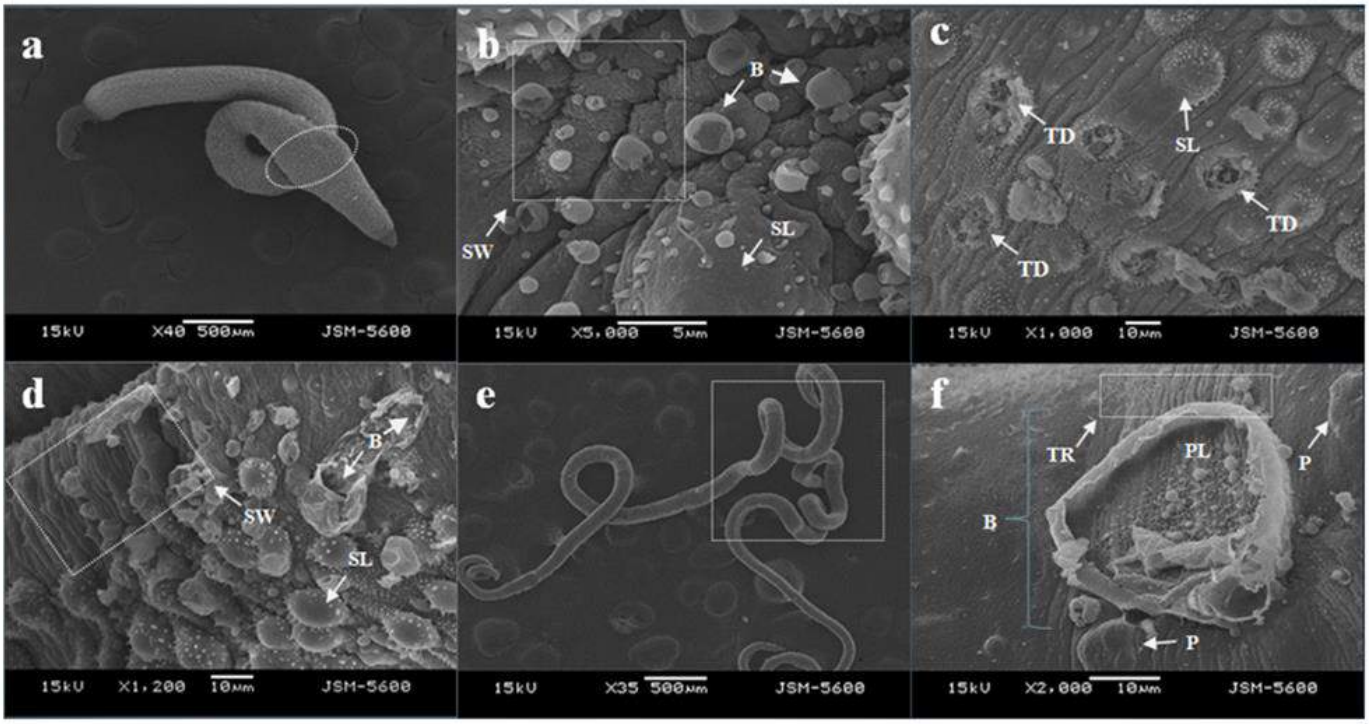


Fig. 5 (**a – f**) SEM images of adult *S. mansoni* worms exposed for 24 h at concentrations of 100 µM of divaricatic acid. (**a**, x40) Male worm exhibiting dorsal curvature with dilatation region in its posterior portion. (**b**, x5000) Tegument showing edema (SW) region with juxtaposition of transverse parallel folds, presence of small bubbles (B) and regions of tubercles with loss of spines (SL). (**c**, x1000) Tubercular damage (TD), with tegument erosion and exposure of the subtegumentar layer and spine loss (SL). (**d**, x1200) Tegument with extensive edema region (SW), large bubbles (B) ruptured with exposure of the subtegumentar layer and expressive number of tubercles presenting loss of spines (SL). (**e**, x35) Two female worms exhibiting dorsoventral curvature with contortion areas (highlighted) in their posterior portions. (**f**, x2000) Female worm's tegument showing bubble rupture (B) with peeling region (PL), tegument retraction regions (TR) and focal points of edema (SW).

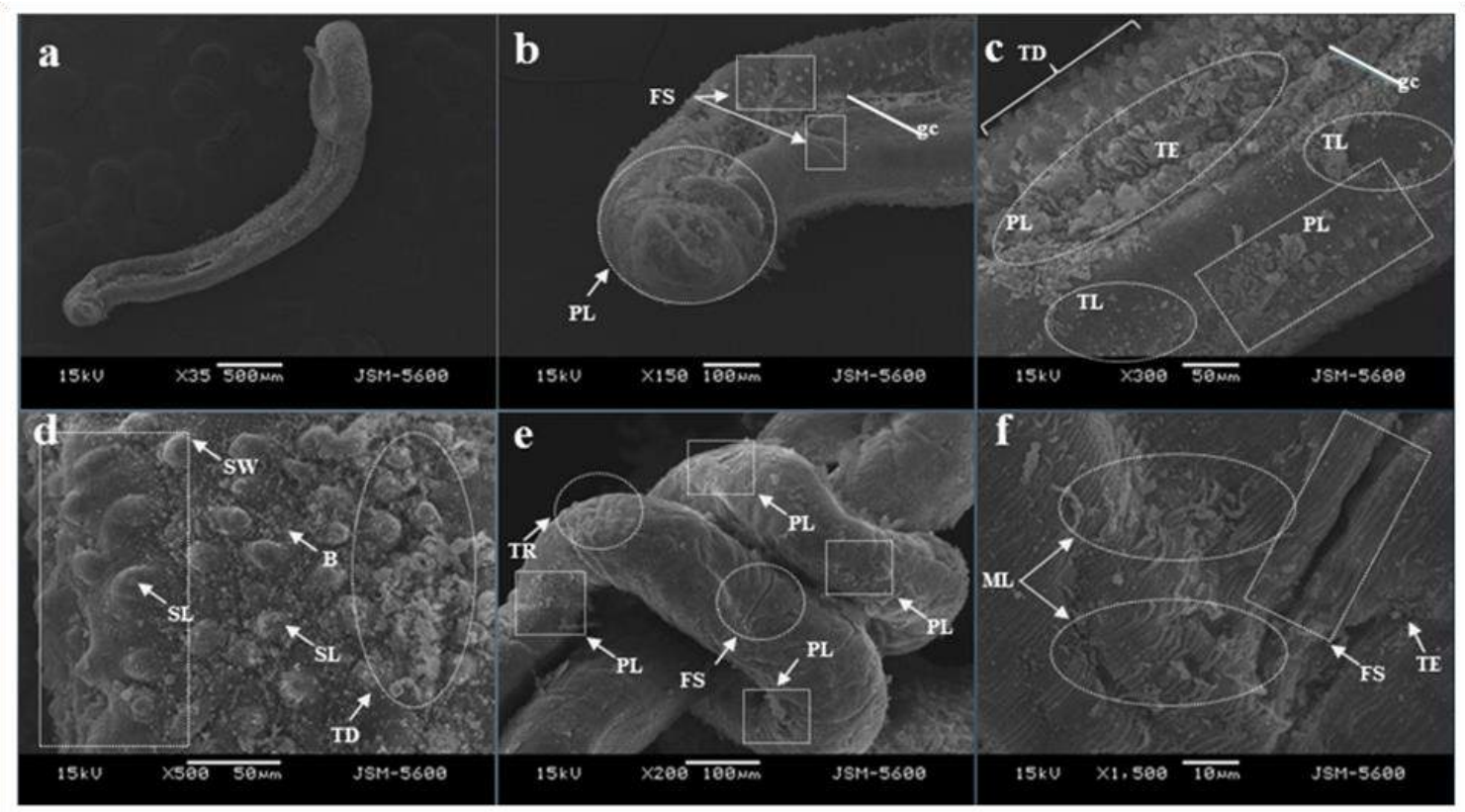


Fig. 6. (**a – h**) SEM images of adult *S. mansoni* worms exposed for 24 h at concentrations of 200 µM of divaricatic acid. (**a**, x35) Male worm exhibiting ventral region, with exposure of the gynecophore canal. (**b**, x150) where it is possible to observe intense peeling (PL) in the region of the oral and ventral suckers, as well as the presence of fissures (FS) near the gynecophore canal (gc). In closer approximation (**c**, x300), an extensive region of erosion (TE) and peeling (PL) of the integument within the gynecophore canal is observed, as well as damage and complete loss of tubercles (TL) in regions adjacent to the gynecophore (gc) canal. (**d**, x500) Extensive edema regions (SW) can be observed, where there was complete loss of tubercular spines (SL), as well as the presence of small bubbles (B) and damage to tubercles (TD). (**e**, x200) Female worms intertwined with tegument presenting regions of desquamation (PL), retraction of tegument (TR) and fissures (FS). (**f**, x500) damage and exposure of muscle fibers (ML) and tegument erosion (TE).

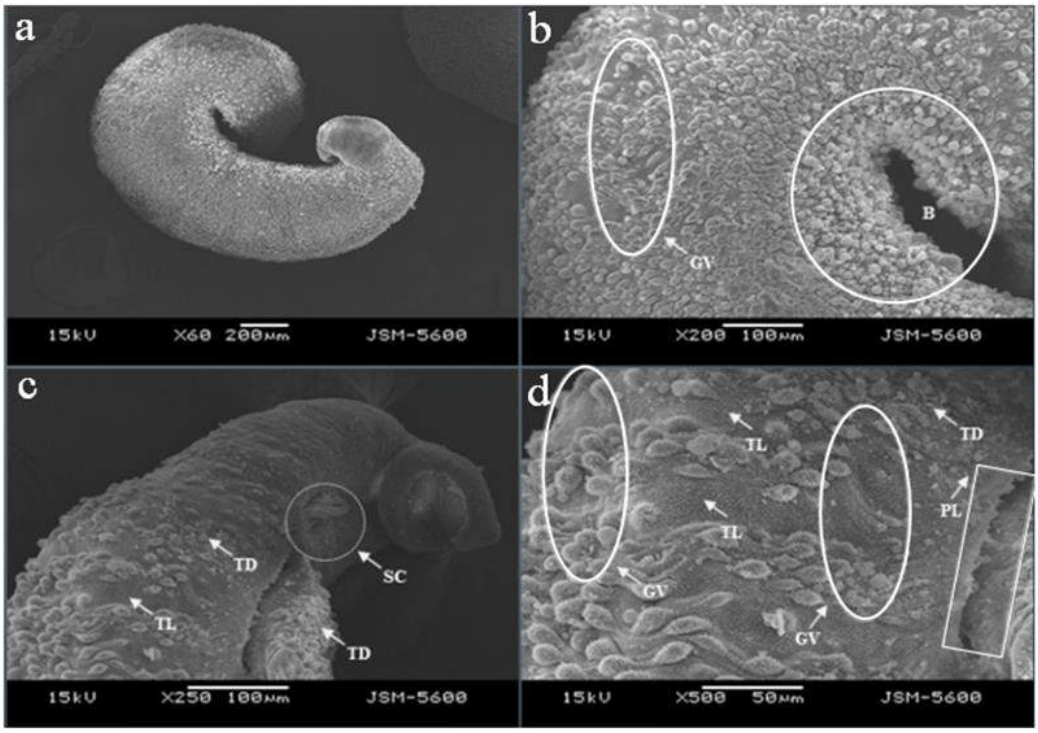
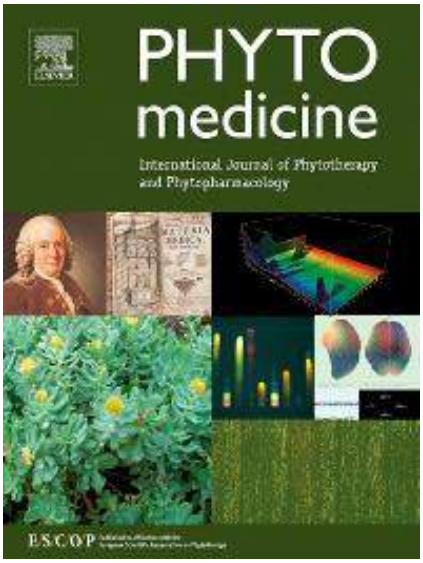


Fig. 7. **(a-d)** SEM images of adult *S. mansoni* worms exposed for 24 h to praziquantel (Positive Control). (**a**, x60) Dorsoventral image of a male worm exhibiting marked contraction of the musculature. (**b**, x200) In evidence, posterior region of the worm presenting bubbles (B) over a large extent of the integument and a region with a slight groove (GV). (**c**, x250) Tegument with damage (TD) and loss of tubercles (TL) and presence of ventral sucker invagination (SC). (**d**, x500) Regions with grooves (GV), loss (TL) and tubercle damage (TD), and peeling (PL) of the integument in the external region of the gynecophore canal.

5 ARTIGO 3 - BARBATIC ACID FROM *Cladia aggregata* (LICHEN): *IN VITRO* SCHISTOSOMICIDAL EVALUATION AND ULTRASTRUCTURAL ANALYSIS AGAINST ADULT WORMS OF *Schistosoma mansoni*

ARTIGO SUBMETIDO AO PERIÓDICO



PHYTOMEDICINE

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Barbatic acid from *Cladia aggregata* (Lichen): *in vitro* schistosomicidal evaluation and ultrastructural analysis against adult worms of *Schistosoma mansoni*

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Abstract

This study evaluated the schistosomicidal effect of barbatic acid, a lichen metabolite, against adult worms of *Schistosoma mansoni*. The *in vitro* schistosomicidal effect was evaluated through the parameters of motility and mortality, cellular viability of the worms and ultrastructural analysis through Scanning Electron Microscopy. To evaluate the cytotoxicity of barbatic acid, a cell viability assay was performed with human peripheral blood mononuclear cells. Barbatic acid showed a schistosomicidal effect after 3 hours of exposure. At the end of 24 h the concentrations of 50 - 200 μM proved lethal to the worms. Motility changes were observed at sublethal concentrations. The IC_{50} values obtained by the cell viability assay for *S. mansoni* was 99.43 μM. Extensive damage to the worm's tegument was observed such as bubbles, peeling, erosion, damage and loss of tubercles and spines, tissue ruptures and fissures. No cytotoxicity was observed on human peripheral blood mononuclear cells. This report provides data showing the schistosomicidal effect of barbatic acid on *S. mansoni*, which included death, motile changes and ultrastructural damage to worms. In addition, barbatic acid was shown to be non-toxic to human peripheral blood mononuclear cells at concentrations which were effective against *S. mansoni*.

Keywords: Anthelmintic activity, Barbatic acid, *Schistosoma mansoni*, Schistosomiasis, Ultrastructural analysis.

Introduction

Schistosomiasis, an infection caused by helminth trematodes of the genus *Schistosoma*, affects approximately 260 million people who are distributed in 78 countries and territories (Colley et al. 2014; World Health Organization 2018). The World Health Organization highlights five species of *Schistosoma* with importance in human medicine; they are *S. hematobium, S. intercalatum, S. japonicum, S. mekongi* and *S. mansoni*, with the latter species being considered the most prevalent, endemic in 54 countries of Africa, the Middle East, the Caribbean and South America (World Health Organization 2018a; World Health Organization 2018b, Crellen et al. 2016).

S. mansoni infection occurs through contact with water contaminated with human fecal material containing helminth eggs. In contact with water these eggs hatch and release miracidia, the infective developmental stage for the intermediate host, which are snails of the genus *Biomphalaria*. Subsequently, after 4-6 weeks, these snails release cercariae, the infective phase for man, the definitive host. Adult worms inhabit the hepatic portal system and mesenteric veins, where the female performs oviposition, and can survive up to 30 years (Gryssels et al. 2006).

Schistosomiasis mansoni is characterized by severe pathological manifestations in the hepato-splenic system and gastrointestinal tract resulting from granulomatous inflammatory reactions. This inflammation can cause hepatosplenomegaly, portal hypertension, ascites and hematemesis of esophageal varices and fibrosis (Gryssels et al. 2006; Aires et al. 2014; Glaser et al. 2015). However, many of those infected can live asymptomatically for years. This clinical condition is important, since this portion of infected individuals has the potential to spread the infection without knowing his/her actual health condition.

Currently, Praziquantel (PZQ) is the only drug used for the treatment and control of schistosomiasis, since there is still no commercialized vaccine (Hotez et al., 2018). Although effective against adult worms, being biosecure and of low cost (Bertão et al. 2012), PZQ, at the recommended doses, does not present therapeutic efficacy against young worms, in addition to not preventing reinfection. Furthermore, mass treatment in endemic countries for the eradication of infection is of great concern to the medical and scientific community, as there are reports of resistance and/or tolerance of *Schistosoma* strains to PZQ (Vale et al. 2017). Thus, it is urgent to search for new drugs of natural, semi-natural and/or synthetic origin that may contribute to the control or eradication of schistosomiasis. Faced with this scenario, the search for new schistosomicidal drugs finds in natural products a promising

source of biomolecules (Godinho et al. 2014; Guimarães et al. 2015). Among these biomolecules, phenolic compounds have shown activity against adult *Schistosoma* worms (Alvarenga et al. 2012; Salloum et al. 2012; 2016; Eraky et al. 2016).

Lichens, a symbiotic relationship between a mycobiont (fungal partner) and photobiont (algae/cyanobacteria), produce a large variety of secondary metabolites, mostly of phenolic nature, which are produced mainly by the fungus and secreted on the surface of their hyphae in amorphous or crystalls forms (Mitrović et al. 2011). The most common lichen compounds are polyketides such as depsides, depsidones, diphenyl ethers and dibenzofurans (Thadhani et al. 2011), which have several biological activities, among them: bactericidal, fungicidal, antiviral, antinociceptive, photoprotective and anti-inflammatory (Maia et al. 2002; Pereira et al. 2010; Zambare and Christopher, 2012; Radice et al. 2016). Barbatic acid, a phenolic lichen substance inserted to the depside group, originates through the esterification of two or more units of orsellinic or β-methyl orsellinic acids (Brandão et al. 2017), and is found in lichens like *Cladina kaubii*, *Usnea flexilis* and *Cladia aggregata*, among other species. It has applications as a herbicide, bactericide, antioxidant, insecticide, antitumor and molluscicide (Ranković and Kosanić 2015; Verma and Behera 2015; Martins et al. 2017; 2016; 2018).

The objective of the present study was to evaluate the schistosomicidal potential, *in vitro*, of barbatic acid, obtained from *Claddia aggregata*, through the analysis of parameters of mortality, motility, cell viability of the worms and tegumentary alterations by scanning electron microscopy, against couples of worms *S. mansoni*. Additionally, the study aimed to evaluate the cytotoxicity of barbatic acid on human peripheral blood mononuclear cells (PBMC).

Materials and Methods

Samples of Cladia aggregata

Lichens of the *Cladia aggregata* species were collected in the Northeast Region of Brazil, in the municipality of Bonito (Pernambuco, Brazil), at the coordinates 08º28'13"S and 35º43'43"W, kept at room temperature (28 ±3 °C), and packed in a desiccator for later identification. The identification of the lichen stalk was performed and an exsiccate deposited in the UFP Herbarium, Department of Botany of the Federal University of Pernambuco (voucher nº 36431).

Extract of C. aggregata*, purification and isolation of barbatic acid*

Barbatic acid was obtained according to the methodology described by Martins et al. (2017). Briefly, a lichen sample (50 g) was subjected to successive extractions with diethyl ether in a Soxhlet apparatus at 30 °C and the solvent was subsequently evaporated at 40 °C. The ether extract obtained was then subjected to successive washes with chloroform in a G4 funnel under pressure. At the same time Thin-Layer Chromatography (TLC) was performed to verify the efficiency of the washes. This process was repeated until only one band was seen in the TLC. For the one-dimensional TLC, in a silica gel 60 plate (Merk® $PF_{254+366}$), the methodology described by Culberson (1969) was used. For elution, the system used was toluene/dioxane/acetic acid (45:12.5:2 - v/v/v) and for the revelation, the gel was sprayed with 10% sulfuric acid followed by heating at 50 °C for 5 min. The bands were observed through the use of UV radiation (254 and 366 nm).

High-performance liquid chromatography (HPLC), ^{1}H *(*^{1}H *NMR) and* ^{13}C *(*^{13}C *NMR) nuclear magnetic resonance and infrared analysis (IR)*

To confirm the isolation of the molecule, the following chemical analyses were performed: the samples that presented only one band on the TLC were then analyzed by HPLC to verify the percentage of purity (> 95%), following the methodology described by Huneck and Yoshimura (1996). A Hitachi Chromatograph (655 A-11, Tokyo, Japan) was used, coupled to a CG437 UV detector set at 254 nm. For the separation, a C-18 reversed-phase column (MicroPack MCH-18) of 300 x 4 mm (Merk® KGaA, Darmstadt, Germany) was used. The samples were injected at a concentration of 1.0 mg mL^{-1} in chloroform (Merk®). The mobile phase consisted of methanol/water/acetic acid (80:19.5:0.5 v/v/v) in isocratic flow. Other analytical parameters were the following: volume of injection 20 mL, attenuation 0.16, pressure 87 atm and flow rate 1.0 mL min^{-1} at room temperature (28 ± 3 ºC).

For the determination of the isolated molecule structure, ^{1}H NMR and ^{13}C NMR were performed using a Varian Unity Plus spectrometer at 400 MHz, 27 °C, with DMSO-D6 as the solvent. Infrared analysis was performed using a Bruker spectrophotometer coupled to a Fourier transformer (IF model 566) using KBr pellets. The analyses were carried out in the Department of Fundamental Chemistry of the Federal University of Pernambuco - UFPE, Brazil.

Ethical considerations, animals and infection by S. mansoni

In vitro experiments were approved by the Animal Experimentation Ethics Committee of the Biosciences Center of the Federal University of Pernambuco (UFPE) (Protocol 23076.036388/2014-11). Female mice (n = 20, Swiss Webster, 35 days old and weighing 28 ± 2 g), were raised and maintained at the Keizo Asami Immunopathology Laboratory (LIKA) of UFPE, according to standard breeding conditions 23 ± 2 ºC, 40-50 % humidity and 12 h light/dark photoperiod) with water and feed (Labina®) *ad libitum*. The mice were infected with the *S. mansoni* Belo Horizonte strain (BH) through percutaneous exposure to 120 cercariae (Olivier and Stirewalt, 1952). The *S. mansoni* BH strain is maintained in the Laboratory of Immunology of Experimental Diseases and Schistosomiasis of LIKA/UFPE, through successive passages in snails *Biomphalaria glabrata* and Swiss Webster mice.

In vitro studies with S. mansoni

After 45 days of infection, the mice were euthanized by cervical dislocation and worms recovered by perfusion of the hepatic portal system and mesenteric vessels with sterile saline (0.9% NaCl w/v) (Smithers and Terry, 1965). Only intact, couples of worms were immediately transferred to Petri dishes with RPMI 1640 culture medium supplemented with 20 mM HEPES, 100 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% fetal bovine serum and washed three times with this medium. Then, two couples were distributed to wells in sterile culture plates with 24 wells. For adaptation, the worms were incubated in a humid atmosphere containing 5% CO_2 at 37 °C for 2 h. After adaptation, barbatic acid, previously solubilized in 1.5% DMSO and supplemented RPMI, was added to final concentrations of 200, 100, 50 and 25 μM. For the negative controls, we used only RPMI medium and RPMI plus 1.5% DMSO. In the positive control, the worms were incubated in 10 μM of PZQ. All experiments were performed in quadruplicate and repeated at least twice (16 pairs of worms per concentration).

Schistosomicidal evaluation criteria

Motility and survival

An inverted microscope (Leica Microsystems, DM IL Wetzlar, Germany) was used to evaluate the motility and survival of couples of worms monitored at 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 integument 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.

Cell viability assay of couples of S. mansoni

The cellular viability of *S. mansoni* adult worms was evaluated after 24 h of incubation in barbatic acid by the cytotoxicity test for 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), according to the methodology described by Aires et al. (2014). Briefly, the worms (two couples per well) were transferred to a 96-well culture dish containing 100 μL MTT (5 mg mL^{-1}) diluted in phosphate buffer, PBS) and incubated for 30 minutes in a dark room at 37 °C. Then, the MTT solution was removed and 200 μL of DMSO were added per well of the plate, with the purpose of dissolving the purple formazan crystals. The plate was kept under stirring for 1 h at room temperature and the optical density measured at 550 nm in a microplate reader (M680, Bio-Rad Laboratories, Inc.). The worms of the positive and negative control groups were submitted to the same test. All experiments were carried out in quadruplicate and repeated at least twice. The significant differences were taken at $p < 0.05$.

Scanning electron microscopy (SEM)

The worms were analyzed by SEM after 24 hours of incubation in barbatic acid. Worms were fixed in 2.5% glutaraldehyde and 4% 0.1 M in sodium cacodylate buffer (pH 7.2) for 12 h at room temperature. After fixation, the samples were washed with this same buffer and post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer for 1 h in

darkness. Subsequently, the samples were washed with 0.1 M phosphate buffer, dehydrated with increasing concentrations of ethanol at 30, 50, 70, 90 and 100% for 10 min. and critical point dried (Hitachi HCP-2, Hitachi, Japan), using CO_2 as a transitional medium. The samples were mounted on small aluminum supports, metallized with gold in a FINE-COAT 1100-JEOL metallizer (Ion Sputter JFC-1100), for 6 min. The specimens were then examined and photographed with a JEOL-JSM-5600 LV Scanning Electron Microscope (Tokyo, Japan).

Ethical considerations and cytotoxicity assay

PBMCs (peripheral blood mononuclear cells) were used for the verification of the toxic effects of barbatic acid on human cells. All donors (healthy individuals, n = 5) of blood samples signed an informed consent form and the study was approved by National Health Council Resolution 466/12 (CAAE) 62919816.2.0000.5208. The culture was performed in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg mL^{-1} streptomycin, at 37 °C with 5% CO_2. The MTT method was used to evaluate cytotoxicity, where the cells were packed in 96-well plates (1 x 10^6 cells / mL) in RPMI 1640 medium and incubated for 24 h (37 °C and 5% CO_2). Barbatic acid was added at an initial concentration of 200 μM and serial dilutions were performed up to the minimum concentration of 1.56 μM, and the plate was subsequently incubated for 72 h. For the negative control, cells were not treated with barbatic acid. Three hours before the final incubation time, 25 μL of MTT (5 mg mL^{-1} in PBS buffer) were added to each well. After 3 h of incubation, the medium was aspirated and 100 μL of DMSO were added to each well, and the optical density was then measured at 450 nm in an ELISA microplate reader (M680, Bio-Rad Laboratories, Inc.) (Albuquerque et al., 2014). The assays were performed in quadruplicate in three independent assays.

Statistical analyses

Significant differences were identified using ANOVA followed by the Student-Newman-Keuls post-test, where $p < 0.05$ was considered significant for all analyses. Statistical analyses were performed using GraphPad Prism 5.0 software for Windows (GraphPad Software, San Diego, CA, USA). The estimated lethal concentration for 50% of the specimens (IC_{50}) was performed by Probit analysis with a 95% confidence interval (CI), using the StatPlus® 2009 Professional software (AnalystSoft, Canada).

Results

Chemical analyses

After the process of obtaining and purifying the barbatic acid, TLC analysis demonstrated a single band with a retention factor of 0.42, a result compatible with the findings of Huneck and Yoshimura (1996). The confirmation of the purity of the isolated compound was performed by HPLC analysis, confirming the purity of the crystals obtained in 99.23% with a retention time of 18.75, as similarly found in a study by Martins et al. (2017). The ^{1}H NMR, ^{13}C NMR and IR analysis (**Table 1**) confirmed the molecular structure of barbatic acid ($C_{19}H_{20}O_7$) (**Fig. 1**).

Changes in cellular motility and viability of couples of worms **S. mansoni** *induced by barbatic acid*

Table 2 shows motility scores of couples of *S. mansoni* incubated in barbatic acid after 3, 6, 12 and 24 h. At all observation intervals, worms from negative control groups, incubated only in RPMI or RPMI medium with 1.5% DMSO, showed no differences in motility and cell viability. These worms showed typical movements, exhibiting peristalsis of the internal organs, moving suckers, which adhered to the bottom or sides of the culture plate; score 3. Thus, we chose to express the cell motility and viability results of worms incubated in RPMI with 1.5% DMSO. After 3h, 81.25% of the worms incubated at 200 μM showed movements only at the extremities or at only one of the extremities (anterior or posterior regions), with absence of peristalsis of the internal organs and suckers not adhered (score 1), and 12.5% showed complete absence of motion and integument with or without changes in coloration; score 0. At 6 h of exposure, 100% mortality was reported at the concentration of 200 μM while 50% of the worms showed reduced movements throughout the body, peristalsis of internal organs and suckers; score 2, when incubated at the concentration of 100 μM. At 12 h, 81.25% and 43.75% of worms incubated at concentrations of 100 and 50 μM, respectively, exhibited score 2. After 24 h, 56.25% and 18.75% of worms exhibited score 0 and 43.75% and 81.25% score 1, when incubated at 100 and 50 μM, respectively. Significant changes in the motility of worms incubated in PZQ are observed after 3 h, however only 6.25% degenerate to score 0 after 6 h. After 24 h, PZQ is capable of causing 87.5% mortality of worms.

In couples of worms *S. mansoni*, barbatic acid reduced significantly ($p < 0.001$) mitochondrial viability and, consequently, cell viability, by reducing formazan crystal formation with IC_{50} (95% CI) of 99.43 μM (98.07 - 100.8 μM). Our results show a reduction in cell viability of 19.33%, 27.69%, 53.68% and 86.45% for the concentrations of 25, 50, 100 and 200 μM, respectively, when compared to the negative control (**Fig. 2**). In addition, in the comparison with the positive control (PZQ), which caused 70.51% reduction in viability of *S. mansoni*, the concentration of 200 μM was more effective in eliminating the parasite ($p < 0.01$).

Ultrastructural alterations of couples of worms* S. mansoni *induced by barbatic acid

Negative control group worms, incubated for 24 h in RPMI medium alone or RPMI medium with 1.5% DMSO, exhibited body topography and ultrastructural aspects within normal morphological parameters. Thus, we chose to express SEM results of worms incubated in RPMI with 1.5% DMSO. **Figure 3** shows coupled (**Fig. 3a-b**) and male (**Fig. 3c-d**) and female (**Fig. 3e-f**) worms of *S. mansoni*. In **Fig. 3a**, it is possible to observe the ventrolateral curvature of coupled worms, where the female (fw) is inserted in the gynecophore channel (cg) of the male worm (mw). In **Figs. 3b**, **3c** and **3e** in an approximate view of the previous figure, the oral (os) and ventral (vs) suckers of male and female worms are observed in detail. The **Fig. 3d** shows the dorsal region of the male worm's tegument, characterized by the presence of tubercles (tb), spines (sp), dome-shaped papillae (dp) and parallel transversal folds (paf). In **Fig. 3e**, the female worm's tegument is seen with the presence of numerous parallel transversal folds (paf), **Fig. 3f**.

After 24 h of incubation, at the lowest evaluated concentration of barbatic acid, 25 μM (**Fig. 4a-c**), it was possible to see in male worms (**Fig. 4a**) the appearance of small bubbles (B), which were diffusely distributed (**Fig. 4b-c**) and the loss of spines (SL) in some tubercles (**Fig. 4c**). When incubated at 50 μM (**Fig. 4d - f**), the male worms exhibited tegumentary damage characterized by the presence of bubbles (B), peeling and tegumentary projections (P) in the middle dorsal region, focal tegument erosion with tubercle loss and loss of spines (SL) in some tubercles (**Fig. 4e-f**). At these two concentrations, 25 and 50 μM, female worms presented preserved tegumentary morphology. At 100 μM, the worms are slightly curved dorsoventrally (**Fig. 5a**). Male worms exhibited invagination (SC) of the ventral sucker and slight peeling (PL) of the oral sucker (**Fig. 5b**), areas of tegument retraction (TR) with displacement and juxtaposition of tubers and transverse parallel folds and areas presenting

tubercular damage (TD) in the dorsal region (**Fig. 5c**). Also, in the dorsal region, extensive tegument destruction (EDT) was found, with exposure of the subtegumentary layer (STL), bubbles (B), erosion (TE) and tubercle destruction (TD) with areas of peeling (PL) (**Fig. 5d-e**). The tegument of female worms showed focal peeling (PL) (**Fig. 5f**). Worms incubated at the concentration of 200 μM (**Fig. 5g-l**) presented more significant tegumentary damage. In the male worms we observed edema of oral sucker (ED), fissures (FS), peeling (PL) and tissue rupture (BK) (**Fig. 5g**). In the dorsal region, extensive tegumentary damage (EDT) was found (**Fig. 5i-j**), characterized by areas with loss (TL) and tubercle degeneration (TD) with loss of spines (SL), loss of parallel transversal folds and dome papillae, presence of bubbles (B), areas of peeling (PL) and focal regions of erosion with subtegumentary layer exposure (STL). In female worms, tegumentary retraction (TR) with groove (GV) formation was seen (**Fig 5k**), as well as regions with extensive erosion (ET) with the presence of bubbles (B), exposure of subtegumentary tissue (STL) and areas of retraction (TR) with displacement of parallel transversal folds (**Fig. 5l**).

Positive control group worms, incubated for 24 h in PZQ, exhibited strong shortening and dorsoventral contraction (**Fig. 6a**) and the anterior region retracted with a reduction in the number of tubercles (**Fig. 6b**). Along the integument, the tubercles were juxtaposed with the loss of spines (**Fig. 6c**) and numerous bubbles were seen (**Fig. 6d**).

Barbatic acid does not present cytotoxicity against PBMC

On human peripheral blood mononuclear cells (PBMCs), barbatic acid did not show significant levels of cytotoxicity at all concentrations tested (1.56 - 200 μM) when compared to the control group, and it was not possible to define an IC_{50} (IC_{50} > 200 μM) for the concentrations evaluated.

Discussion

The absence of alternative drugs and a biosecure vaccine (Santini-Oliveira et al., 2015) makes praziquantel (PZQ) currently the only pharmaceutical alternative for fighting schistosomiasis. In addition to the emergence of resistant and/or tolerant strains of *Schistosoma*, this scenario also becomes unfavorable because of the way PZQ is marketed. Distributed as a racemate, PQZ is composed of equivalent proportions of two stereoisomers, where only one is biologically active, which contributes to the large size of the tablet and its

bitter taste, which hinders adherence to treatment, especially among children (Gouveia et al., 2018). In this way, the search for alternative compounds is an urgent necessity.

Natural products have a remarkable variety of chemical compounds with diverse structures and characteristics, presenting themselves as an excellent sources of new biomolecules with applications in chemistry, biology and medicine (Shen 2016). Several classes of molecules of natural origin have already been tested *in vitro* on *S. mansoni* worms, among these terpenes, phenolic compounds, quinones, flavonoids and peptides (Moraes 2015). However, this is the first report of the use of a depside of lichen origin in schistosomicidal evaluation. Our results demonstrate that barbatic acid, a phenolic metabolite extracted from the lichen *Cladia aggregata*, presented schistosomicidal activity *in vitro* against *S. mansoni* couples, as it was able to cause alterations in motility, tegumentary damage and to reduce cell viability of worms.

According to the results, barbatic acid caused 100% mortality at a concentration of 200 μM after 6h of incubation, while at a concentration of 100 μM caused 56.25% of mortality after 24h. These results corroborate the IC_{50} value (99.43 μM), determined from the cell viability assay of worms after 24 h of incubation. Recently, Martins et al. (2017), explored the schistosomicidal potential of barbatic acid against *S. mansoni* cercariae, reporting mortality and changes in motility. Other phenolic compounds have already demonstrated the promising schistosomicidal potential of this chemical group, as is the case of β-lapachone, derived from lapachol extracted from trees of the genus *Handroanthus*, which demonstrated *in vitro* activity on *S. mansoni*, causing mortality and tegumentary changes (Aires et al. 2014). Allam and Abuelsaad (2014), when evaluating the schistosomicidal effect *in vitro* on *S. mansoni* of hesperidin, a phenolic compound extracted from citrus fruits, reported 100% lethality at the concentration of 200 μg mL^{-1}.

The tegument of adult *S. mansoni* worms is a protective cuticle that plays an important role in defense, selective absorption of drugs and nutrients, metabolism, osmoregulation, excretion and tissue repair and proliferation (Aires et al. 2014; El-Shabasy et al. 2015). Although several drugs cause tegumentary damage in *S. mansoni*, this analysis has received little attention in the search for new therapeutic alternatives for schistosomiasis. Currently however, the study of tegumentary damage is of great importance for the elucidation of the mechanism of action of schistosomicidal agents, with the parasite's tegument being the target of drugs such as atorvastatin, mefloquine and thioxo-imidazolidine compounds (Bertão et al. 2012).

In our work, barbatic acid caused progressive tegumentary damage in a dose-dependent manner, causing bubbles, peeling, erosion, damage and loss of tubercles and spines, tissue ruptures, fissures, tegumentary retraction, tubercles and displacement of transverse parallel folds and formation of grooves. These alterations caused by barbatic acid are of great importance, since deep tissue damage exposes surface antigens of the parasite, leading to recognition by the immune system of the host, and thus completing the mechanisms for the elimination of the worm (Cupit and Cunningham 2015).

Similar results to the findings of this study were found by Oliveira et al. (2014), who evaluated the *in vitro* schistosomicidal activity of the dichloromethane and aqueous extracts of *Baccharis trimera* on adult worms of *S. mansoni*. The authors obtained 100% lethality of the parasites at a concentration of 130 μg mL^{-1}, after exposure of 24 h for both extracts, observing tegumentary damage such as peeling and/or destruction of tubercles and spines, several bubbles between the tubercles and damage in the oral and ventral suckers. Ultrastructural damage was also found by Lorsuwannarat et al. (2013), after evaluating the schistosomicidal activity of pumblagin on adult *S. mansoni* worms. Changes such as swelling and erosion of tubercles with loss of spines, peeling of suckers and damage to the gynecophore canal with exposure of the adjacent basal membrane were observed. Similarly, Araújo et al. (2019), when evaluating the schistosomicidal activity of the potassium salt of usnic acid, another phenolic lichen compound, observed dose-dependent progressive damage to the *S. mansoni* integument, such as the appearance of bubbles, edema, muscle contraction, erosion and disintegration of the tubercles and tegument of male and female worms.

The molecular mechanism of action of PZQ is known to act by destabilizing Ca^{2+} homeostasis and neuromuscular function of the worm through voltage-dependent Ca^{2+} channels (Greenberg 2005), compromising the structure and function of the tegument, spasm and paralysis of the underlying muscles, and culminating in the eventual death of the parasite (Lorsuwannarat et al. 2013). The mechanisms of action of various lichen compounds, among them barbatic acid, are still not well understood. However, a conjecture for the reduction of *S. mansoni* motility demonstrated by barbatic acid is its depressant effect on the production of leukotrienes, by 5-lipoxygenase (5-LO), which uses arachidonic acid as a precursor. It was demonstrated that barbatic acid was able to inhibit the production of leukotriene B_4 (LTB_4), with an IC_{50} of 7.8 μM. A decrease in this activity was observed as free carboxyl groups were esterified, suggesting an important participation of this group for the inhibitory effect (Kumar and Müller 1999). According to studies by Salafsky and Fusco (1987), cercariae, schistosomules and adult worms of *S. mansoni* produce several eicosanoids, among them

LTB_4, which actively participates in the cercarian penetration process. In an *in vitro* evaluation, an increase in cercarian penetration was observed after increasing levels of leukotrienes, as well as their decrease at low levels of this eicosanoid through the insertion of a 5-LO inhibitor (Fusco et al. 1986; Fusco et al. 1987). The same was observed by Zhou et al. (2009) in a study with *S. japonicum*, where the authors confirm the production of LTB_4 by these worms, as well as the production of putative receptors for leukotrienes B_4, cysteinyl leukotrienes and prostaglandins E2 and F2, suggesting that these prostaglandins could have an important role in the physiology of schistosomes and also in the interaction between host and parasite

There are still few studies involving evaluations of cytotoxicity of barbatic acid on non-tumor cells. This is the first report, also, of the cytotoxic effect of barbatic acid on human PBMC cells. In the present study, barbatic acid did not demonstrate significant levels of toxicity at any of the concentrations evaluated, demonstrating specificity to *S. mansoni*.

Conclusion

This is a preliminary evaluation of the schistosomicidal potential of barbatic acid against *S. mansoni* and human non-tumor cells. In all bioassays, barbatic acid was effective in eliminating the parasite, causing, also, significant ultrastructural damage, within concentrations not toxic for PMBCs. This study presents significant results, introducing the chemical group of depsides, a class still little studied, as possible antiparasitic agents. However, further evaluations are needed to elucidate the mechanism of action involved in elimination and ultrastructural damage to parasites.

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Conflict of Interest

All authors declare that there is no conflict of interest.

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Table 1 ^{1}H e ^{13}C NMR and IR analysis of barbatic acid

Infrared (KBr) approximate vibrational assignments		^{1}H (400 MH_Z, DMSO-d6) NMR spectroscopic data		^{13}C (400 MH_Z, DMSO-d6) NMR spectroscopic data	
Position	ν (cm^{-1})	Position	δ_H (*J* in H_Z) ppm	Position	δ_C (in ppm)
5	3100 ν (C-H) aromatic	8’	1.96 s (CH_3)	1	110.31 (C)
8	2947 ν_{as} (CH_3)	8	1.97 s (CH_3)	2	152.04 (C)
9	2930 ν_s (CH_3)	9’	2.45 s (CH_3)	3	106.62 (C)
7	1718 ν (C=O) ester	9	2.54 s (CH_3)	4	161.46 (C)
	1659 ν (C=O) phenyl ester conjugated	4	3.72 s (OCH_3)	5	107.11 (C)
7’	1630 ν (C=O) carboxylic acid	5	6.56 s (H)	6	139.28 (C)
1 and 2*	1574 ν (C=C) aromatic	5’	6.65 s (H)	7	168.96 (C)
	1522			8	8.26 (C)
8	1444 δ_{as} (CH_3)	2	10.74 s (OH)	9	22.99 (C)
9	1338 δ_s (CH_3)			1’	161.42 (C)
7**	1280 ν (C-O) chain ester			2’	111.75 (C)
	1150			3’	159.79 (C)
7’	1209 ν (C-O) carboxylic acid			4’	116.22 (C)
				5’	139.36 (C)
				6’	116.03 (C)
				7’	173.38 (C)
				8’	9.30 (C)
				9’	23.34 (C)
				4’’	56.00 (C)

* Positions 1 and 2 correspond to the two absorptions of the double bond of the aromatic ring
** Position 7 corresponds to the two absorptions of the carbonyl of the ester.

Table 2 Motility scores of adult *S. mansoni* worms treated with barbatic acid at different concentrations and exposure times.

Groups	Percent of worms (%) in motility scores after incubation															
	3h				6h				12h				24h			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Control				100				100				100				100
PZQ																
10 µM		81.25	18.75		6.25	93.75			62.5	37.5			87.5	12.5		
Barbatic Acid																
200 µM	12.5	81.25	6.25		100				100				100			
100 µM				100			50	50		6.25	81.25	12.5	56.25	43.75		
50 µM				100				100		6.25	43.75	50	18.75	81.25		
25 µM				100				100				100			25	75

Note: percentage values of 32 worms (16 pairs of worms per concentration) per group.
Score 3 = present typical movements, exhibitinhg 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 and suckers. Slow peristalsis of the organs.
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.

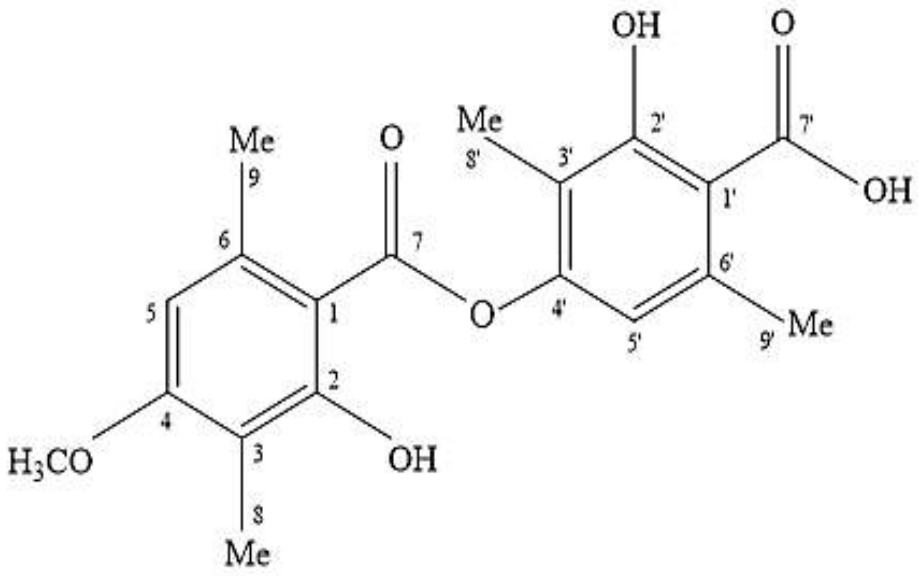

Fig. 1. Molecular structure of barbatic acid

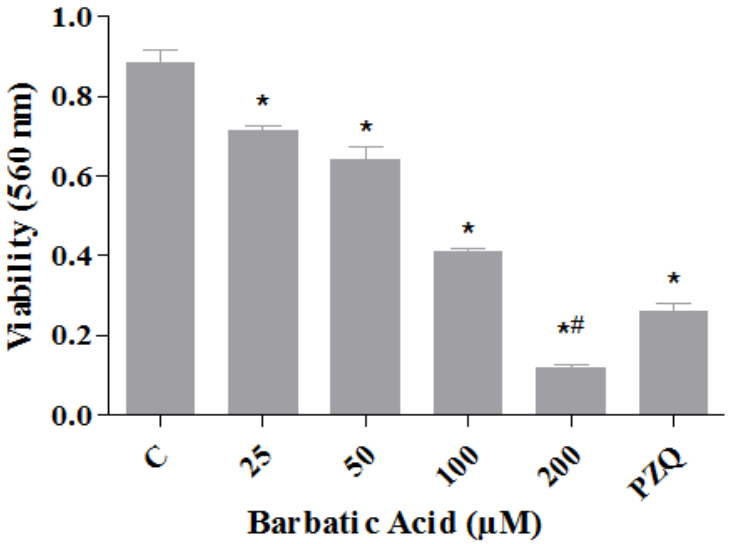

Fig. 2. *In vitro* effects of barbatic acid (25, 50, 100 and 200 µM) on the cell viability of *S. mansoni* couples after 24 h incubation in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Viability was expressed as the mean ± SD of the absorbance values. * p <0.001 when compared to the negative control group (C, RPMI medium with 1.5% DMSO). #p <0.001 when compared to the positive control (PZQ).

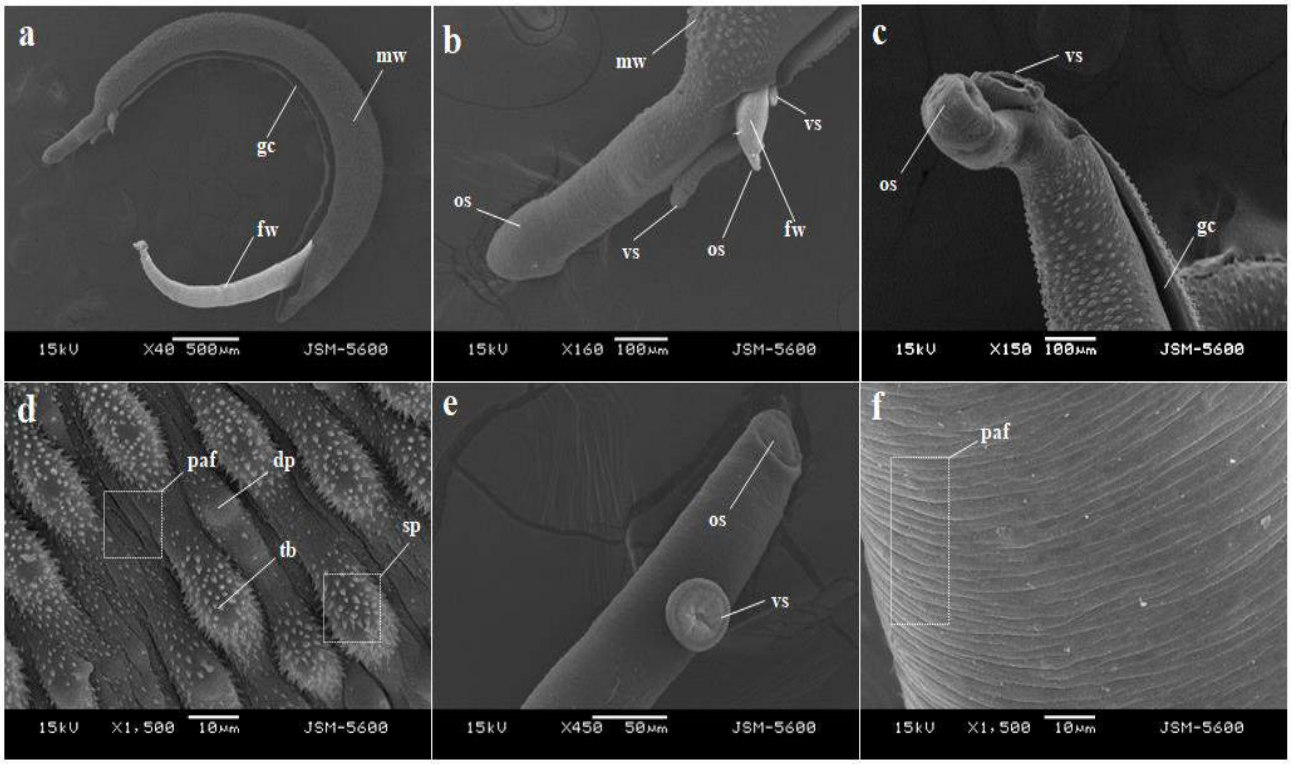

Fig 3. (a-f) SEM images of adult male and female worms of *S. mansoni* from the Negative Control Group (RPMI with 1.5% DMSO) after incubation for 24 h. (**a**, x40) Coupled worms, where the male (mw) exhibits ventrolateral curvature, oral (s) and ventral (vs) suckers, with female (fw) inserted in its gynecophore channel (gc); (**b**, x160) Enlarged view of **a**, showing the anterior region of the coupled worms exhibiting the well preserved tegumentary morphology; (**c**, x150) Anterior region of male worm, exhibiting oral (s) and ventral (vs) suckers; (**d**, x1500) Tegument of the dorsoventral region, showing tubercles (tb) with spines (sp), dome-shaped papillae (dp) and well preserved parallel folds of the tegument (paf); (**e**, x450) Anterior region of the female worm exhibiting the oral (s) and ventral (vs) suckers, with well preserved morphology; (**f**, x1500) Detailed view of the female worm's tegument showing parallel-arranged folds (paf).

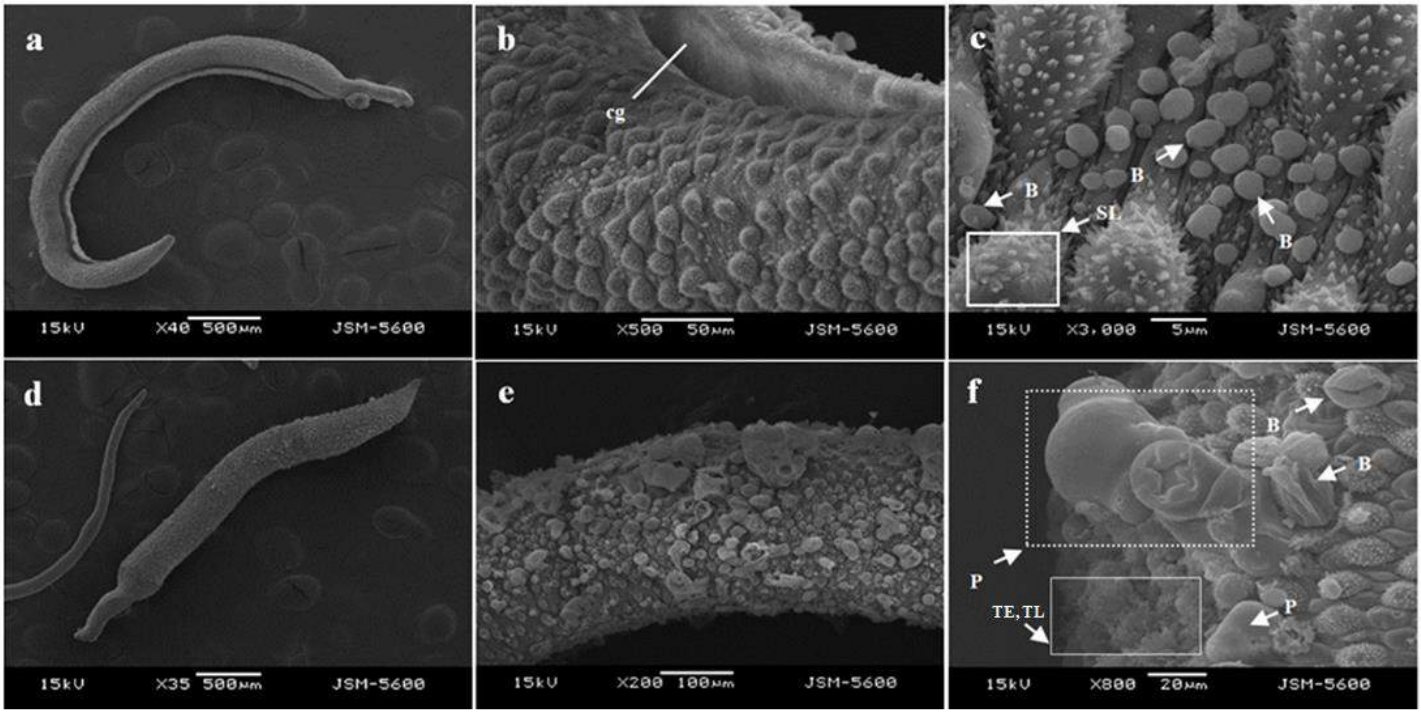

Fig 4. (a-f) SEM images of adult *S. mansoni* worms exposed for 24 h to barbatic acid at concentrations of 25 µM (a-c) and 50 µM (d-f). (a, x40) Male worm exhibiting dorsoventral curvature; (b, x500) median view of the worm, along the region near the gynecophore canal (gc), showing slight tegumentary alterations; (c, x3000). It is possible to observe the loss of spicules (SL) in some tubercles and the presence of small bubbles (B); (d, x40) dorsal region of male worm; (e, x200) where it is possible to observe extensive tegumentary damage and the presence of large bubbles distributed throughout the tegument; (f, x800) projections of the tegument can be observed in some regions (P), as well as tegumentary erosion (TE) with tuber loss (TL).

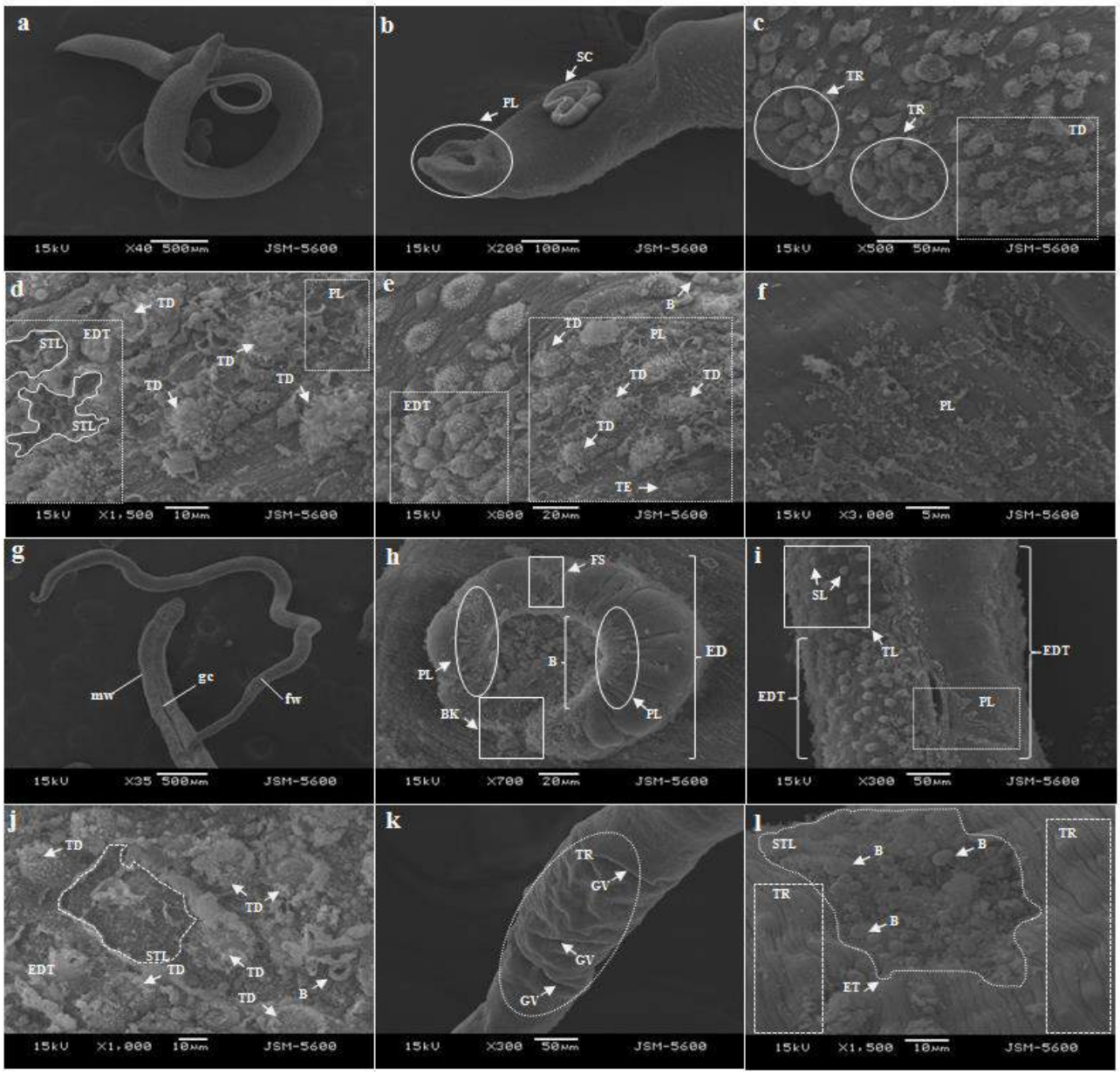

Fig 5. (a-l) SEM images of adult *S. mansoni* worms exposed for 24 h to barbatic acid at the concentration of 100 µM (a-f) and 200 µM (g-1). (a, x40) Coupled worms exhibiting dorsal curvature; (b, x200) Anterior region of male worm showing invagination of the ventral sucker and slight peeling of the oral sucker; (c, x500) Areas of retraction of the tegument (TR) with displacement and juxtaposition of tubercles and transverse parallel folds, and areas presenting tubercular damage (TD); (d, x1500) Male worm tegument showing extensive destruction of the tegument (EDT) with exposure of the subtegumentary layer (STL), several damaged tubercles (TD) and peeling region (PL); (e, x800) Male worm tegument presenting extensive tegument destruction (EDT), areas of peeling (PL) with small bubbles (B) and several damaged (TD) and eroded (TE) tubercles; (f, x3000) Female worm tegument showing extensive peeling area (PL). (g, x35) partly coupled worms exhibiting ventral curvature, wherein the female is partially inserted in the gynecophore canal of the male; (h, x700) Oral sucker of male worm presenting edema (ED), a fissure (FS), a break (BK) in its lower portion, areas of peeling (PL) and several bubbles (B); (i, x300) Ventral region of male worm, showing areas of extensive tegument destruction (EDT), areas with tubercles (TL) and spines (SL) loss and peeling (PL); (j, x1000) Male worm tegument showing extensive destruction of the characteristic tegumentary morphology (EDT), with subtegumentary layer

exposure (STL), presence of bubbles (B) and damaged tubercles (TD); (k, x300) Female worm showing intense tegument retraction (TR) with the presence of several grooves (GV); (l, x1500) Female worm tegument presenting tegmental retraction (TR) with displacement of the transversal folds, tegumentary erosion region (ET) with exposure of subtegumentary tissue (STL) and several bubbles (B).

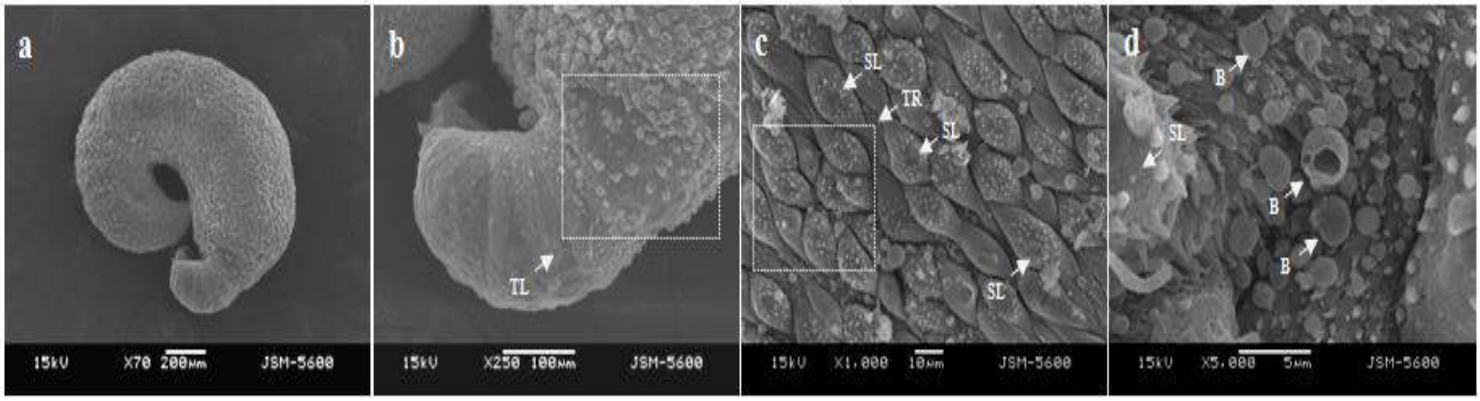

Fig 6. (a-d) SEM images of *S. mansoni* adult worms exposed for 24 h to Praziquantel (Positive Control). (a, x70) Dorsoventral image of a male worm exhibiting sharp contraction and shortening; (b, x250) Detail of the anterior region of the worm with tubercle loss (TL); (c, x1000) Tegument showing tubercles with loss of spines (SL) and regions of tegument retraction (TR) with crowding of tubers; (d, x5000) Presence of bubbles (B) evenly distributed throughout tegument.

6 CONCLUSÕES

- Foi possível obter os extratos dos liquênicos etéreos de *R. aspera*, *C. texana* e *C. aggregata*, bem como isolar e purificar os ácidos divaricático (98.45% de pureza) e barbático (99.23% de pureza);
- O extrato etéreo de *R. aspera* se mostrou eficiente na avaliação de atividade moluscicida para caramujos adultos, causando 100% de letalidade na concentração de 9.5 μg mL^{-1}, com CL_{50} de 7,0 μg mL^{-1};
- A redução da fecundidade foi observada em *B. glabrata* após exposição ao extrato etéreo de *R. aspera* em todas as concentrações avaliadas, atingindo 73.11% na maior concentração avaliada (8.5 μg mL^{-1});
- Alterações citomorfológicas foram observadas nos hemócitos de *B. glabrata* expostos ao extrato de *R. aspera* em todas as concentrações avaliadas, como binucleações, trinucleações, núcleos reniformes, micronucleações, vacúolos citoplasmáticos e células apoptóticas;
- Um aumento dose-dependente na frequência das principais alterações hemocitárias foi observado, com elevação de 0,46%, 0, 36% e 36,46% na maior concentração avaliada (8.5 μg mL^{-1}), para as alterações de binucleação, micronúcleo e apoptose, respectivamente;
- O extrato etéreo de *R. aspera* apresentou efeito embriotóxico sobre todos os estágios embrionários de *B. glabrata* avaliados, apresentando CL_{90} de 22,78; 24,23; 16,63 e 16,03 μg mL^{-1}, para as fases de blástula, gástrula, trocófora e véliger, respectivamente;
- A atividade cercaricida do extrato etéreo de *R. aspera* foi observada a partir da concentração de 5 μg mL^{-1} após 60 minutos de exposição;
- O extrato etéreo de *R. aspera* se apresentou atóxico no bioensaio de toxicidade ambiental com *Artemia salina* nas concentrações efetivas sobre *B. glabrata* e cercárias de *S. mansoni*;
- Os ácidos divaricático e barbático causaram alterações de motilidade em vermes adultos de *S. mansoni* em todas as concentrações avaliadas após 24 horas de exposição, apresentando letalidade nas concentrações de 50 a 200 μM para o ácido barbático e nas concentrações de 100 e 200 μM para o ácido divaricático;

- Foi observada a diminuição da viabilidade celular de *S. mansoni* após exposição de 24 horas, com IC_{50} de 100.6 μM para o ácido divaricático e 99.43 μM para o ácido barbático;
- Os ácidos barbático e divaricático causaram alterações ultraestruturais extensas em *S. mansoni*, como a presença de bolhas, perda de espinhos e tubérculos, erosão tegumentar, descamação, edema e exposição de camadas subtegumentares;
- No bioensaio de citotoxicidade com PBMC, os ácidos barbático e divaricático não apresentaram toxicidade significativa nas concentrações efetivas sobre *S. mansoni*.

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APÊNDICE 1 - BARBATIC ACID OFFERS A NEW POSSIBILITY FOR CONTROL OF *Biomphalaria glabrata* AND SCHISTOSOMIASIS

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Barbatic Acid Offers a New Possibility for Control of *Biomphalaria Glabrata* and Schistosomiasis

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Abstract: This study evaluated the biological activity of an ether extract and barbatic acid (BAR) from *Cladia aggregata* on embryos and adult mollusks of *Biomphalaria glabrata*, cercariae of *Schistosoma mansoni* and the microcrustacean *Artemia salina*. The ether extract and BAR were obtained by successive extractions with diethyl ether. The obtained extracts were analyzed using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), proton nuclear magnetic resonance (^{1}H-NMR) and infrared (IR) spectroscopy. The results demonstrated that the ether extract exerted embryotoxic effects at 50 and 100 μg/mL and molluscicidal effects at 20 and 25 μg/mL. BAR exhibited no embryotoxicity, and its molluscicidal concentration was equal to that of the ether extract. However, after 60 min of exposure, 1 μg/mL BAR presented cercaricidal activity against the parasite *S. mansoni* at the second larval stage. Neither substance induced toxicity against *A. salina*. These results indicate the potential molluscicidal activities of the ether extract and BAR against *B. glabrata* and *S. mansoni* cercariae. In addition to these effects, there was a lack of toxicity against the aquatic environment and no damage to the biota, indicating the potential of these products for large-scale control and/or eradication of schistosomiasis.

Keywords: *Cladia aggregata*; embryotoxicity; molluscicidal activity; environmental toxicity; lichen substances; mollusks

1. Introduction

Human schistosomiasis, a parasitic disease caused by trematode worms of the *Schistosoma* genus, is one of the most prevalent and debilitating parasitoses among neglected tropical diseases. It has been estimated that approximately 261 million people require treatment for schistosomiasis in 78 countries in Africa, Asia and South America [1,2].

In Brazil, the snail *B. glabrata* is the most important vector for schistosomiasis and is associated with high rates of disease infection and transmission. This species can be found in aquatic environments,

where the snails spawn and release cercariae of *S. mansoni* (infective larval stage) [1,3], which are generally eradicated with synthetic chemicals, such as niclosamide, as recommended by the World Health Organization (WHO) [4]. However, although niclosamide at low concentrations efficiently eliminates snails and cercariae at all phases of the life cycle, it is both light-sensitive and toxic to fish, amphibians and aquatic plants [5]. In addition, the costs associated with the application of niclosamide are high, and the snails can develop resistance to this synthetic molluscicide [6]. Thus, natural products from plants and/or other organisms [7,8] could be considered promising sources of novel substances with molluscicidal activity.

In nature, organisms utilize various nutritional strategies, including lichenization, which is a symbiotic association between a fungus (usually an ascomycetes), an algae (often of the Chlorophyceae class), and/or a cyanobacterium. This arrangement ensures that the lichen can benefit from secondary metabolites, which have extensive biological uses [9]. Because of their different biological properties, including antimicrobial [10,11], anti-tumor [12–15], antiherbivore [16], insecticidal [17], and molluscicidal activities [18], secondary metabolites, such as polyphenolic compounds from lichens, have been used in folk medicine since ancient times [19].

Asplund et al. [20] reported that secondary metabolites from thalli lichen have an important ecological function in preventing slugs and land snails from feeding on these species. According to Lawrey [21], the food preference of some land mollusk species for thalli free of depsides and dibenzofurans (e.g., stictic and usnic acids, respectively) might be related to palatability, indirect effects on intestinal microbiota, antibiosis, and the direct toxicity of these substances to mollusks.

For this reason, it was hypothesized that barbatic acid (BAR) might also exert effects on aquatic mollusks, such as adult *B. glabrata* or larval *S. mansoni.* BAR is an important depside that acts on the photosynthesis photosystem II [22], inhibits non-redox reactions during the synthesis of leukotrienes (LTB4), suppresses keratinocyte proliferation [23], and demonstrates bactericidal activity against *Staphylococcus aureus* [11] as well as antitumor activity [24,25]. However, there are no reports of any molluscicidal activity of this metabolite.

In this context, phenolic compounds from lichens are a promising source for biomolecules. Certain polyphenolic compounds present in higher plants have been shown to exhibit molluscicidal properties against different species of mollusks [26,27]. Therefore, given their similar metabolic origins, we hypothesized that both the ether extract of and BAR from *C. aggregata* (lichen) efficiently act on embryos and adults of *B. glabrata* and *S. mansoni* cercariae. In addition to testing this hypothesis, we further investigated whether these molecules exert toxic effects on the environment through bioassays using brine shrimp (*Artemia salina*) as a bioindicator.

2. Results

2.1. Chemical Analysis

Lichens possess several characteristic phenolic chemotypes whose occurrence can vary depending on season and collection site. This phenotypic plasticity is a typical feature of *C. aggregata* and depends primarily on environmental conditions. For this reason, different chemotypes, such as stictic, constictic, norstictic and cryptostictic acids, can be found. In contrast, in Brazil, BAR is the dominant substance found in the species, which is consistent with the findings obtained in this study. Chromatographic analysis of BAR ($C_{19}H_{20}O_7$) from *C. aggregata* revealed a R_f value of 0.43 for TLC, whereas HPLC revealed a R_T value of 19.74 min and a purity of 96.6%. The ether extract contained both BAR (RT 19.7 min) and STI (2.8 min) at respective concentrations of 93% and 0.2%, as well as other minor compounds that remain unidentified. ^{1}H-NMR and ^{13}C-NMR data confirmed that the molecular structure of BAR was in accordance with the one reported by Martins et al. [11] (Figure 1).

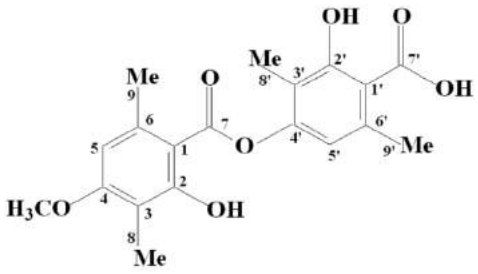

Figure 1. Structure of BAR.

2.2. Toxicity of the Ether Extract of And BAR from C. aggregata on Embryo and Adult Mollusks

Significant embryotoxic activity was demonstrated for the ether extract of *C. aggregata* (*** $p < 0.0001$, F = 380.3). Statistically significant differences were obtained at concentrations higher than 10 µg/mL (*** $p < 0.0001$) compared with the negative control (water and 0.5% ethanol). Furthermore, 100% mortality was observed at 50 µg/mL (Figure 2). No significant difference was detected between the concentrations of 10, 10.5, 11, 11.5, 12, and 12.5 µg/mL and the negative control or between 50 µg/mL (95% CI of diff: −7.729 to 7.729) and the positive control. However, a significant difference was found between 15 and 50 µg/mL (95% CI of diff, −62.73 to −47.27, *** $p < 0.0001$) and between 20 and 50 µg/mL (95% CI of diff, −57.73 to −42.27, *** $p < 0.0001$). The LC_{50} of the ether extract on embryos was 19.9 µg/mL, whereas BAR exerted no toxic activity.

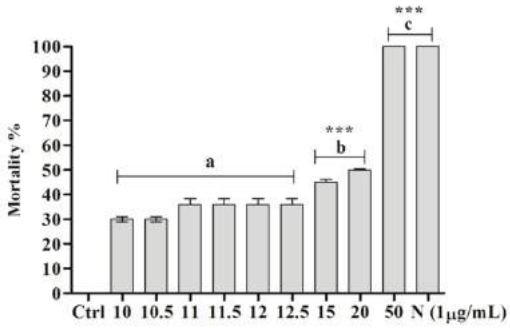

Figure 2. Effect of ether extract (µg/mL) of *C. aggregata* on *B. glabrata* embryos: Ctrl—negative control (0.5% ethanol + filtered and dechlorinated water); N—niclosamide (1 µg/mL). The asterisks (***) indicate significant differences ($p < 0.0001$) compared with the negative control. The letter "**a**" indicates that the treatments presented no statistically significant differences between groups, the letter "**b**" indicates significant differences compared with **a**, and the letter "**c**" indicates that the treatments (50 µg/mL and N) did not present significant differences between groups.

Treatment with the ether extract revealed significant differences for concentrations higher than 10.5 µg/ mL (ANOVA, $p < 0.0001$, F = 35.68) compared with the control (Ctrl). At 20 µg/mL, there was 100% mortality (*** $p < 0.0001$) compared with the Ctrl, demonstrating the efficiency of the ether extract at lower concentrations. However, Tukey's test showed no significant difference among concentrations of 11, 11.5, 12 and 12.5 µg/mL. Among the treated groups, statistically significant differences were found between 10.5 and 20 µg/mL (95% CI of diff, −4.790 to −1.8, *** $p < 0.0001$), 11 and 20 µg/mL (95% CI of diff, −4.457 to – 1.5, *** $p < 0.0001$), 11.5 and 20 µg/mL (95% CI of diff, −4.124 to −1.2, *** $p < 0.0001$), 12 and 20 µg/mL (95% CI of diff, −4.124 to −1.2, *** $p < 0.0001$) and 12.5 and 20 µg/mL (95% CI of diff, −3.790 to −0.8, *** $p < 0.0001$). Furthermore, no statistically significant difference was detected between 20 or 25 µg/mL and the positive control (niclosamide), proving the efficiency of the ether extract (Figure 3A). The LC_{50} value for the extract was calculated as 11.9 µg/mL.

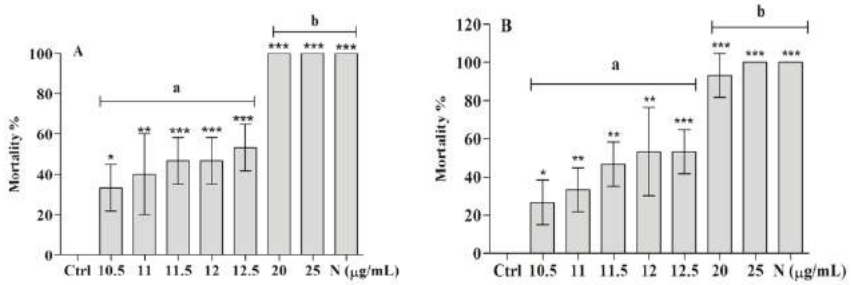

Figure 3. Molluscicidal activity (μg/mL) of the ether extract (**A**) and BAR (**B**) against *B. glabrata* snails. Ctrl—negative control (0.5% ethanol + filtered and dechlorinated water); N—niclosamide (1 μg/mL). The significance levels of the differences compared with the negative control (ANOVA) are indicated with * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.0001$). The letters "**a**" and "**b**" indicate that the groups did not and did present significant differences (Tukey's test, *** $p < 0.0001$), respectively.

Similar to the ether extract, BAR showed substantial molluscicidal activity against snails (ANOVA, *** $p < 0.0001$, F = 27.78) at a concentration as low as 11 μg/mL (* $p < 0.05$) compared with the negative control. However, the greatest molluscicidal activity was observed at 25 μg/mL, which showed 100% lethality. The LC_{50} value for BAR was 11.9 μg/mL. Among the treated groups, no significant difference was detected between the concentrations of 10.5, 11, 11.5, 12 and 12.5 μg/mL (Figure 3B). Significant differences were found between 10.5 and 20 μg/mL (95% CI of diff, −99.71 to −33.63, *** $p < 0.0001$), 10.5 and 25 μg/mL (95% CI of diff, −106.4 to −40.29, *** $p < 0.0001$), 11 and 20 μg/mL (95% CI of diff, −93.04 to −26.96, *** $p < 0.0001$), 11 and 25 μg/mL (95% CI of diff, −99.71 to −33.63, *** $p < 0.0001$), 11.5 and 20 μg/mL (95% CI of diff, −79.71 to −13.63, ** $p < 0.01$), 11.5 and 25 μg/mL (95% CI of diff, −86.37 to −20.29, *** $p < 0.0001$), 12 and 20 μg/mL (95% CI of diff, −73.04 to −6.960, * $p < 0.05$), 12 and 25 μg/mL (95% CI of diff, −79.71 to −13.63, ** $p < 0.01$), 12.5 and 20 μg/mL (95% CI of diff, −73.04 to −6.960, * $p < 0.05$), and 12.5 and 25 μg/mL (95% CI of diff, −79.71 to −13.63, ** $p < 0.01$). There was no significant difference between 20 and 25 μg/mL and the positive control, proving the efficiency of both concentrations compared with niclosamide.

Mollusk spawns were treated with the ether extract and BAR, and those that survived were collected and analyzed until the first generation hatched (F1). Interestingly, although 66% of the mollusks treated with 10.5 μg/mL ether extract survived, they were not able to spawn. The same result was observed following exposure to 11, 11.5, 12.5 and 20 μg/mL BAR, clearly demonstrating that these substances interfered with the mollusk spawning process (Table 1).

Table 1. Percentage viability and inviability of snail embryos (F1) that survived treatment with the ether extract and BAR.

Substance	Concentration (μg/mL)	No. of Fecund Embryos *	Viability ♦ (%)	Inviability ■ (%)
Ctrl †	–	379	0.5	99.5
Ctrl ‡	0.5%	300	1.5	98.5
Ether extract	10.5	–	–	–
	11	44	6.8	93.2
	12.5	26	0	100
BAR	10	38	0	0
	11	–	–	–
	11.5	–	–	–
	12	25	4	96
	12.5	–	–	–
	20	–	–	–

Legend: † Filtered and dechlorinated water; ‡ 0.5% ethanol + filtered and dechlorinated water; * number of embryos produced; ♦ hatched embryos; ■ malformation and/or mortality; – no spawning.

2.3. Toxicity of BAR from C. aggregata to S. mansoni Cercariae

Table 2 shows the partial lethality of BAR on *S. mansoni* cercariae in relation to exposure time. Cercaricidal activity was first detected after exposure to a concentration of 0.25 μg/mL for 30 min (+), and more than 50% lethality (++) was obtained after exposure to a concentration of 0.5 μg/mL for 60 min. Complete elimination of cercariae (+++) was observed after exposure to 1 μg/mL for 60 min. The time required for complete elimination of cercariae decreased with increasing concentration, with 30 min being needed at a concentration of 10 μg/mL and 15 min at a concentration of 100 μg/mL.

Table 2. Cercaricidal activity of BAR against *S. mansoni*.

Concentration (μg/mL)	15 min	30 min	60 min	120 min
Ctrl	−	−	−	−
0.25	−	+	+	+
0.5	−	+	++	++
1	−	++	+++	+++
10	++	+++	+++	+++
100	+++	+++	+++	+++
N	+++	+++	+++	+++

Legend: Complete elimination of cercariae (+++), elimination of more than 50% of cercariae (++), elimination of less than 50% of cercariae (+), and absence of lethality (−). Ctrl (0.5% ethanol + filtered and dechlorinated water), N (niclosamide).

During the treatment, there were changes in engine behavior, such as atypical rotation and vibrations. Specifically, the cercariae exhibited slow rotation around their own axes, creeping and different intensities of contractions that increased as the concentration of BAR was increased. These findings highlight the dose-dependent effect of the substance. Figure 4 shows images highlighting the significant differences among the treatments: in the negative control group (A), the cercariae presented normal rotation and vibration motility accompanied by preservation of the body and tail, whereas the treatment group (B) showed separation of the body and cercariae tail, and the positive control group (C) resulted in cercariae death. However, niclosamide did not cause separation of body and tail, as was observed in the group treated with BAR.

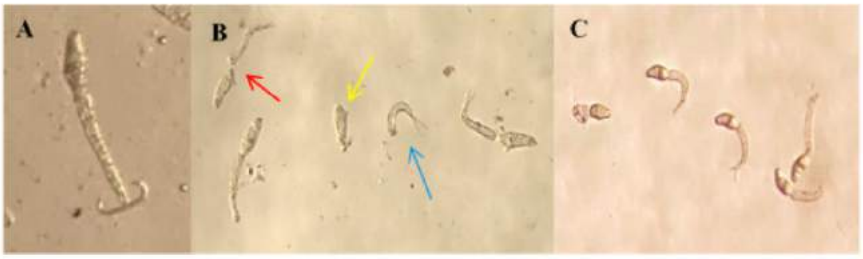

Figure 4. Cercariae of *S. mansoni* exposed to BAR. (**A**) Image of cercariae treated with 0.5% ethanol and filtered water, showing preservation of the body and tail; (**B**) Image of cercariae exposed for 30 min to 1 μg/mL BAR, showing a split between the body and tail (red arrow); an individual body (yellow arrow) and tail (blue arrow) are also displayed; (**C**) Image of dead cercariae after exposure to niclosamide (1 μg/mL). 40× magnification.

Figure 5 displays the lethality of BAR at the end of the experiment (120 min), at which time all concentrations showed significant differences compared with the Ctrl. The average lethality values obtained for 0.25, 0.5, 1, 10 and 100 μg/mL were 46 ± 4.58 ($p < 0.0001$), 62 ± 12.0 ($p < 0.0001$), 100 ($p < 0.0001$), 100 ($p < 0.0001$), and 100 ($p < 0.0001$), respectively. The LC_{50} was calculated as 0.45 μg/mL.

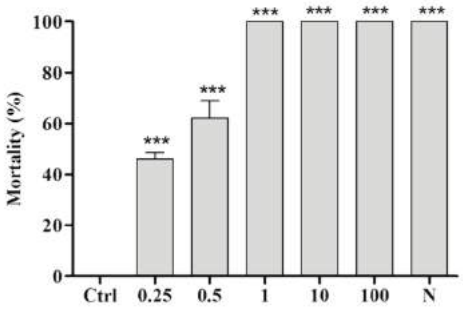

Figure 5. Cercaricidal activity of BAR against *S. mansoni* at the end of the 120-min exposure period. Ctrl (0.5% ethanol and filtered water); N (niclosamide at 1 μg/mL). The results were compared with the Ctrl; *** $p < 0.001$.

2.4. Toxicity of the Ether Extract of and BAR from C. aggregata against A. salina

Ecotoxicity assays are important for establishing safe environmental parameters regarding the use of xenobiotics. For this reason, we tested both the ether extract and BAR on the environmental bioindicator *A. salina*. The results showed that both products from *C. aggregata* were nontoxic against *A. salina* at all tested concentrations, with $p = 0.0904$ and F = 1.967 for the extract (Figure 6A) and $p = 0.1710$ and F = 1.601 for BAR (Figure 6B). However, at 100 μg/mL, the effects of the extract were significant ($p < 0.05$, 95% CI of diff, 3.418 to −0.08152).

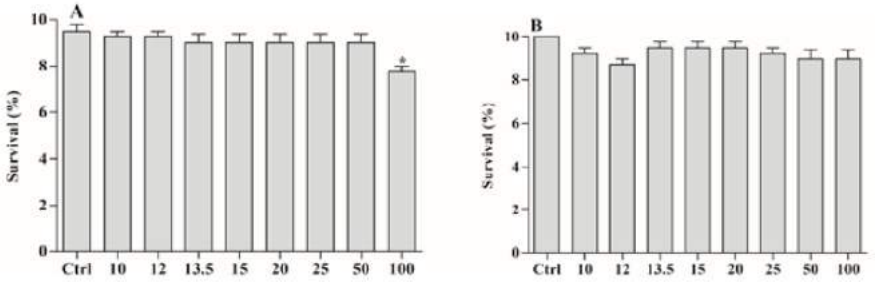

Figure 6. Toxicity of ether extract (**A**) and BAR (**B**) on *Artemia salina*. The ctrl was 0.5% ethanol and seawater. * $p < 0.05$.

3. Discussion

Research correlating lichen substances and schistosomiasis vectors is still novel because few reports on these topics have been published. In this context, Martins et al. [18] evaluated the molluscicidal activity of potassium usnate in a pioneering study of this type of biological activity and demonstrated that *B. glabrata* shows embryotoxicity and molluscicidal activity at 1 μg/mL and 10 μg/mL, respectively. However, contrary to potassium usnate, the BAR tested in this study did not exert any effect on embryos. In contrast, the molluscicidal activity of BAR was significant (20 μg/mL) and in accordance with the standards recommended by the WHO [4] because it eliminated 90% of the same mollusk population, like potassium usnate. Even though potassium usnate is a lichen phenolic modified to a salt form, its solubility in water is higher than that of BAR, a property that could enhance its ability to induce mortality compared with that of BAR. Because potassium usnate is a salt that can be structurally modified from dibenzofurane derivative, a lichen phenolic (usnic acid). The findings obtained for the organic extract were significant, showing that it could serve as a molluscicide and that it exhibits embryotoxic activity at low concentrations (as low as 10 μg/mL), even though it does not cause 100% mortality. According to HPLC analysis, the BAR content in the extract is over 90%; thus, we expected to obtain a higher LC_{50} for BAR than for the extract. Additionally, we analyzed the

chemistry of the ether extract, which contains other substances capable of potentializing its biological effects on mollusks. Importantly, the concentrations of lichen substances used in this study are lower than those found in some plant extracts, showing the efficiency of BAR on mollusks. For example, a chloroform extract of *Solanum siniacum* displayed molluscicidal activity at 64.4 μg/mL [28].

Ecologically, lichen substances play a key role in thallus maintenance and act as protectors of small, mobile herbivores (insects, snails and mollusks) [20]. Lawrey [21] reported that snails of the species *Pallifera varia* (Hubricht) avoid feeding on lichen species such as *Xanthoparmelia cumberland* (Gyelnik) Hale, which contain usnic, norstictic and stictic acids, and *Huilia albocaerulescens* (Wulfen) Hertel, which produce constictic and stictic acids. The food preferences of invertebrates were reported by Fröberg et al. [29] and Benesperi and Tretiach [30], who disclosed that snails preferentially feed on different parts of lichens. Gauslaa [31] described the food preferences of lichens that do not have secondary metabolites, even though metabolites can be extracted using the acetone rinsing method, which indicates these substances cause some type of toxicity to these animals. Lawrey [21] believes that in addition to reducing the palatability of lichens, the substances show direct toxicity or indirect antibiotic effects on the gut microbiota of predator organisms. However, we believe that this effect cannot be extended to our findings because neither the ether extract nor BAR was toxic to *A. salina*, an environmental bioindicator species, and these preliminary results of environmental toxicity with *A. salina* indicate that BAR could be non-toxic or less toxic than niclosamide. Based on these findings, these substances are potentially safe for the environment.

Studies aiming to identify molluscicidal agents should consider methods to not only suppress parasite vectors but also combat the infectious stage of *S. mansoni*. A substituted pyridine pentahydrate (2,6-dimethyl-3,5-carboxydiethyl-4-phenylpyridine) was assayed against *B. glabrata* and *S. mansoni* cercariae and showed promising results, eliminating 90% of mollusks at 36.43 μg/mL and 100% of cercariae at 4 μg/mL within 30 min of exposure [32]. However, essential oil extracted from *Piper cubeba* L. was effective against cercariae of *S. mansoni* at 200 μg/mL [33], a concentration above that recommended for environmental applications according to the WHO [4]. Our results demonstrate the efficacy of BAR, which eliminated 100% of cercariae at a concentration of 1 μg/mL, the same concentration used for the niclosamide positive control.

A study of *Glinus lotoides* (Molluginaceae) showed that exposure of *S. mansoni* cercariae to an aqueous extract of the plant at a concentration of 18.7 μg/mL resulted in motility alterations that directly influenced the potential penetration of cercariae into mice and reduced the parasite load by 91.2% [34]. Similar motility alterations were observed in this the present study, suggesting that cercariae exposed to BAR at sub-lethal concentrations had reduced infectivity. Separation of body and tail has also been observed in other studies [35,36] and is likely a result of weaknesses in the tail structure. Although the mechanism of action has not yet been elucidated, it is believed that this process is associated with the actions of certain substances on a special connective structure between the body and tail [37].

Ravaglia et al. [38] indicated the importance of screening biologically active compounds for toxicity and verified the toxicity of extracts from *C. aggregata* on *A. salina*. Their results (LC_{50} = 690.6 μg·mL^{-1}) differ from ours, which revealed that neither BAR nor the ether extract exhibited toxicity at lower doses, although the ether extract at a concentration of 100 μg/mL exhibited toxicity, potentially due to the combination of substances present in the extract. According to Ahti et al. [39], *C. aggregata* contains stictic, constictic, norstictic, and cryptostictic acids.

4. Materials and Methods

4.1. Extract Production and Purification of BAR from Cladia aggregata (Sw.) Nyl.

C. aggregata (50 g) was collected in Bonito, Pernambuco, Brazil at the coordinates 08°28′13″ S and 35°43′43″ W Gr. on 6 November 2010. A sample was deposited in the Herbarium UFP of the Universidade Federal de Pernambuco/UFPE, Brazil (Voucher No. 36431). BAR was obtained through

successive extractions with a Soxhlet apparatus (30 °C) using diethyl ether. To isolate and purify BAR, the extract was successively washed with chloroform in a G4 funnel under pressure. BAR was obtained at high purity (>95%), as monitored through thin-layer and liquid chromatography. The molecular structure was determined by ^{1}H-NMR and IR. Additionally details related to the extraction and purification of BAR and the preparation of organic extracts are provided by Martins et al. [11].

4.2. Mollusks

Pigmented mollusks of the species *B. glabrata* (Say, 1818) were obtained from São Lourenço da Mata, Pernambuco, and were reared in the Radiobiology Laboratory of the Department of Biophysics and Radiobiology of the Federal University of Pernambuco through successive generations. The mollusks were selected according to the diameters of their shells (10–14 mm) and were maintained in plastic tanks with filtered and dechlorinated water (pH 7.0, ±25 °C) and fed fresh lettuce (*Lactuca sativa*).

4.3. Embryotoxic Activity of the Ether Extract of and BAR from Cladia aggregata

To test embryotoxicity, we collected egg masses (n = 100) in the blastocyst phase and examined their viability using a stereomicroscope (Leica MZ6, Leica Microsystems, Wetzlar, Germany). The embryos were deposited on Petri dishes (6 mm) and treated with the ether extract from *C. aggregata* or BAR at concentrations of 1, 10, 10.5, 11, 11.5, 12, 12.5, 15, 20 and 50 μg/mL solubilized in ethanol (0.5%) at a final volume of 10 mL per plate for 24 h. The control groups were the following: 1 μg/mL niclosamide (N; positive control; Bayluscide, Bayer, Leverkusen, Germany), ethanol (0.5%; negative control) and filtered and dechlorinated water (Ctrl, negative control). The embryos were evaluated for malformation and/or mortality [40], and the experiment was performed in triplicate.

4.4. Molluscicidal Activity of the Ether Extract of and BAR from Cladia aggregata

To determine molluscicidal activity, a population of 400 mollusks was pre-selected and maintained in isolation for five days to confirm sexual maturity. Groups of mollusks (n = 5) were transferred to small aquariums (500 mL), where they were treated with either the ether extract or BAR (dissolved in 0.5% ethanol) from *C. aggregata* at 1, 10, 10.5, 11, 11.5, 12, 12.5, 20, 25, 50 and 100 μg/mL for 24 h. Two negative controls were used: filtered water and ethanol (0.5%) plus filtered and dechlorinated water (Ctrl). For the positive control, niclosamide (N) was used at 1 μg/mL. After a 24 h exposure period, the animals were washed in distilled water, left untreated, fed fresh lettuce (*L. sativa*) and observed for 96 h. The mollusks that survived the treatment were monitored, and the egg masses were again recorded and analyzed to evaluate the fertility and fecundity of the snails. Retraction of the mollusks into their shells and/or the release of hemolymph were used as indicators of death [41]. The experiment was performed in triplicate.

4.5. Cercaricidal Activity of the Ether Extract of and BAR from C. aggregata

Pigmented and infected *B. glabrata* mollusks were placed in a 200 mL beaker, submerged in distilled water and exposed to artificial light (60 W) for 2 h until the elimination of cercariae was achieved. To assay toxicity, approximately 100 cercariae suspended in distilled water were placed in a glass container and exposed to 2 mL of BAR solubilized in ethanol (0.5%) at 0.25, 0.5, 1.0, 10 and 100 μg/mL. The cercariae were observed using a stereomicroscope (Wild M3B, Heerbrugg, Switzerland) at intervals of 15, 30, 60 and 120 min after exposure and evaluated using the following parameters: complete elimination of cercariae (+++), elimination of more than 50% of cercariae (++), elimination of less than 50% of cercariae (+) and absence of lethality (−). Niclosamide at a concentration of 1 μg/mL and 0.5% ethanol plus filtered and dechlorinated water (Ctrl) were used as positive and negative control groups, respectively. The bioassay was performed in triplicate. The assaying of various time intervals allowed us to observe atypical rotations and vibrations of cercariae during the experiments, and the results were registered through images obtained with a digital camera coupled

to a stereomicroscope (40×). After 120 min, the number of dead cercariae was counted, and the LC_{50} was calculated.

4.6. Environmental Toxicity Assays with Artemia salina

A. salina cysts (25 mg) were incubated in filtered seawater under artificial light at a temperature of 30 °C ether extract or BAR at concentrations of 1, 10, 12, 13.5, 15, 20, 25, 50 and 100 mg/mL solubilized in seawater and 0.5% ethanol. The tubes were incubated for 24 h, and survival was determined for each treatment. Two groups of negative controls were included: filtered seawater and 0.5% ethanol (Ctrl) plus filtered seawater. The experiment was performed in quadruplicate [42].

4.7. Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Significant differences were established through analysis of variance (ANOVA) and Tukey's test at $p < 0.05$. The lethal concentrations (LC_{50}) for the embryos, mollusks and *A. salina* were calculated via Probit analysis with 95% confidence intervals using StatPlus 168 2006 software (Soft Analyst, Vancouver, BC, Canada).

5. Conclusions

The presented results expand the available knowledge regarding the application of active metabolites for the control of schistosomiasis vectors. These findings should be of great interest to researchers in the fields of public health and environmental preservation because the studied molluscicidal substances showed great biological potential. In addition, their characteristics were in accordance with WHO standards, and they were nontoxic to the environment, as demonstrated through bioassays with *A. salina*.

From an environmental point of view, BAR appeared to be more efficient than the corresponding ether extract. Although BAR does not exert any effect on embryos, it is capable of inhibiting adult mollusks because it impedes both laying and spawning in addition to causing cercariae malformation and/or mortality. These findings led us to hypothesize that the substance can directly or indirectly act against all stages of the *S. mansoni* life cycle, indicating that BAR should be further explored in additional studies.

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Author Contributions: Mônica Cristina Barroso Martins and Ana Maria Mendonça Albuquerque de Melo conceived and designed the experiments with *B. glabrata*; Monique Costa Silva and Luanna Ribeiro Santos Silva performed the experiments with *B. glabrata*; Mônica Camelo Pessoa de Azevedo Albuquerque and André Lima Aires conceived and designed the experiments with *S. mansoni* larvae; Hianna Arely Milca Fagundes Silva performed the experiments with *S. mansoni* larvae; Emerson Peter da Silva Falcão analyzed the data; Mônica Cristina Barroso Martins and Eugênia C. Pereira wrote the manuscript; and Nicácio Henrique da Silva analyzed the data and revised all of the experiments.

Conflicts of Interest: The authors declare no conflicts of interest.

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Data article

Data set of the toxic effects of divaricatic acid depside on *Biomphalaria glabrata* and *Schistosoma mansoni* cercariae ☆

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ABSTRACT

In this study, the molluscicidal and antiparasitic activities of divaricatic acid was evaluated, targeting the mollusc *Biomphalaria glabrata* and cercariae of the helminth *Schistosoma mansoni*. Divaricatic acid showed high toxicity against both adult snails (5.5 µg/mL) and embryos (20 µg/mL after 6 h of exposure). Similar activity was observed in *S. mansoni* cercariae after only a short exposure time. The divaricatic acid proved to be a promising substance for the control of the snail *B. glabrata*, an intermediate host of schistosomiasis, as well as the cercariae of the pathogen.

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Specifications Table

Subject area	*Chemistry, Biology*
More specific subject area	*Natural products biochemistry*
Type of data	*Table and figures*
How data was acquired	Stereoscopic microscope (Wild M3B, Heerbrugg, Switzerland)
Data format	Analyzed
Experimental factors	Divaricatic acid purification from *Ramalina aspera* lichen
Experimental features	Molluscicidal and embryotoxic activities on snails of the *Biomphalaria glabrata* species and the cercaricidal activity on *Schistosoma mansoni* of divaric acid were evaluated.
Data source location	*Recife, Brazil.*
Data accessibility	Data found in this article

Value of the data

- The data detail the embryotoxic, molluscicidal and cercaricidal activities of divaricatic acid, facilitating the correlation between the different tests and their concentrations, aiming to eliminate the vector in its different phases, and the etiologic agent of schistosomiasis in the same concentrations.
- The data provide a better understanding of the inviability/mortality information of *B. glabrata* used to obtain the lethal concentrations (LC_{10}, LC_{50} and LC_{90}) present in the original article.
- A more detailed view at the end of the analysis of the cercaricidal activity is provided by the expression of numerical data.

1. Date

The data presented in this paper provide results related to embryotoxicity of divaricatic acid on *Biomphalaria glabrata* at different exposure times (6, 12, 18 and 24 h) (Table 1), as well as the molluscicidal activity of this compound on adult snails (Fig. 1) in 24 h of exposure. Data concerning the cercaricidal activity (*Schistosoma mansoni*) are shown in Fig. 2, where the percentage of dead organisms is reported at the final time of analysis (2 h of exposure to divaricatic acid).

2. Materials and methods

2.1. Materials

2.1.1. Schistosoma mansoni strain

BH strain, from Belo Horizonte, Minas Gerais, Brazil, maintained in Keizo Assami Immunology of the Federal University of Pernambuco (UFPE), through successive passages in snails of the species *Biomphalaria glabrata* kept in the Department of Tropical Medicine (UFPE).

2.1.2. Biomphalaria glabrata molluscs

Geographical line from São Lourenço da Mata, Pernambuco, Brazil, maintained by successive generations in the Laboratory of Radiobiology of the Department of Biophysics and Radiobiology (UFPE).

2.1.3. Divaricatic acid

Divaricatic acid was obtained from the ethereal extract of *Ramalina aspera* lichen and isolated according to the crystallization methodology of Asahina and Shibata [1] with modifications and its

Table 1
Inviability of *Biomphalaria glabrata* embryos subjected to divaricatic acid at different exposure times (6, 12, 18 and 24 h).

Unviability by exposure period				
Experimental groups (g/mL)	**6 h SD**	**12 h SD**	**18 h SD**	**24 h SD**
Control 1	1.33 1.15	1 0.0	0.6 0.57	1 0.0
Control 2	3.33 1.52	2.33 0.57	3 2.64	2 1.7
Niclosamide	100	100	100	100
Dicaricatic acid				
	0.6 0.57	6.33 1.52	6.33 4.04	10.66 7.3
	1 0.0	9.66 4.61	19.66 3.51	31.33 19.0
	1.66 0.57	11 1.0	32.66 5.77	33.66 28.5
	1.66 2.08	18 6.92	35 8.88	39.33 8.0
	8.33 3.05	25.66 8.38	39.66 4.50	47.33 16.2
	10.33 6.02	36.66 11.68	49 11.53	60.66 9.0
	15 4.35	44.66 9.01	55.33 9.07	67.66 17.1
	19.33 3.78	45.66 4.93	67.33 12.01	72.66 6.8
	25.66 4.04	66.66 12.42	75 17.44	84 15.3
	81.66 14.29	94 5.19	96 6.24	100
	100	100	100	100

Control 1: filtered and dechlorinated water. Control 2: 0.5% DMSO in filtered and dechlorinated water. Niclosamide at a concentration of 1 g/mL. Significant results were compared with control 2.

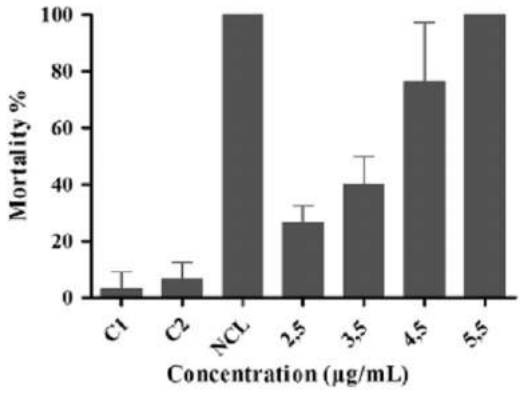

Fig. 1. Mortality of *Biomphalaria glabrata* adult snails exposed to divaricatic acid. Control 1 (C1): filtered and dechlorinated water. Control 2 (C2): 0.5% DMSO in filtered and dechlorinated water. NCL: Niclosamide at a concentration of 1 g/mL.

purity was confirmed by Thin Layer Chromatography [2] and High Performance Liquid Chromatography [3].

2.2. Methods

2.2.1. Embryotoxicity test in B. glabrata

The assay was performed according to the methodology described by Oliveira-Filho and Paumgartten [4]. *B. glabrata* embryos in the blastula stage (n = 100) were exposed to divaricatic acid solubilized in 0.5% DMSO in different concentrations (7.5, 8.0, 8.5, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 15.0 and 20 µg/mL), incubated for 6, 12, 18 and 24 h (25 C 3) and subsequently washed with filtered and dechlorinated water (pH 7.0). The negative control was formed by two groups exposed to filtered and dechlorinated water (Control 1) and 0.5% DMSO solution (Control 2). Niclosamide (Bayluscide, Bayer) was used for the positive control [5], at a concentration of 1 µg/mL. Eight days after exposure, the embryos were analyzed for inviability (malformed embryos or dead) through a stereoscopic

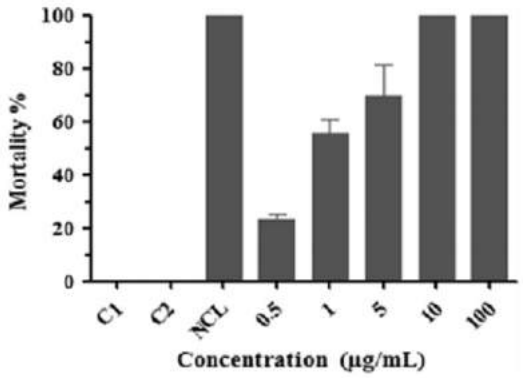

Fig. 2. Mortality of *Schistosoma mansoni* cercariae exposed to divaricatic acid for 120 min. Control 1 (C1): filtered and dechlorinated water. Control 2 (C2): 0.5% DMSO in filtered and dechlorinated water. NCL: Niclosamide at a concentration of 1 g/mL.

microscope and classified into embryos that were hatchlings and inviable (dead or malformed). The experiment was performed in triplicate.

2.2.2. Lethality test in B. glabrata

The assay was performed according to the methodology described by World Health Organization [6]. Adults *B. glabrata* snails were exposed to concentrations of 2.5, 3.5, 4.5 and 5.5 µg/mL of divaricatic acid solubilized with 0.5% DMSO for 24 h (25 C 3). The negative control was formed by two groups exposed to filtered and dechlorinated water (Control 1) and 0.5% DMSO solution (Control 2). Niclosamide (Bayluscide, Bayer) was used for the positive control [7], at a concentration of 1 µg/mL. The snails were observed daily and eight days after exposure, they were analyzed for lethality (absence of body movement, deep retraction into the shell, loss of hemolymph and absence of heartbeat).The test was performed in triplicate.

2.2.3. Lethality test on Schistosoma mansoni cercariae

The assay was performed according to the methodology described by Santos et al. [8] with modifications. Snails of the species *B. glabrata* were exposed for 1 h in artificial light for the release of cercariae. For the test, approximately 100 cercariae were exposed to concentrations of 0.5, 1.0, 10.0 and 100 µg/mL of divaricatic acid. The divaricatic acid was solubilized in 0.5% DMSO. The negative control was formed by two groups exposed to filtered and dechlorinated water (Control 1) and 0.5% DMSO solution (Control 2). Niclosamide (Bayluscide, Bayer) was used for the positive control [9], at a concentration of 1 µg/mL. Afterwards, the cercariae were evaluated and counted for mortality after the 2 h period of exposure. The test was performed in triplicate.

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APÊNDICE 3 - RADIOSENSITIZER EFFECT OF USNIC ACID ON *Biomphalaria glabrata* EMBRYOS

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ORIGINAL ARTICLE

Radiosensitizer effect of usnic acid on *Biomphalaria glabrata* embryos

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ABSTRACT

Purpose: Some phytochemicals have shown the potential of being radiomodifiers, especially phenolic compounds, such as lichenic secondary metabolites. To evaluate the phytochemical usnic acid as a radiomodifier, embryonic cells of molluscs have been used due to their ease of collection, high sensitivity to physical and chemical agents, well-known embryology and low cost for analysis.

Materials and methods: This study aimed to assess the radiosensitizing action of usnic acid on *Biomphalaria glabrata* embryos. Samples were irradiated with 4 Gy of gamma rays from a ^{60}Co source (dose rate 2.906 Gy/h). An acute toxicity test was performed using *B. glabrata* embryos in the blastula stage, in order to determine the toxicity of usnic acid and to establish the lethal Concentration for 50% (LC_{50}). Subsequently, the radiomodifing capacity of usnic acid was estimated using assays with *B. glabrata* embryos.

Results: Irradiation increased the number of non-viable embryos compared to unirradiated controls. Additionally, it was observed that embryos exposed to a non-toxic concentration of usnic acid (0.6 µg/mL) before irradiation showed a further enhancement in non-viable embryos when compared with exposure to ionizing radiation alone.

Conclusion: The results presented here indicate that usnic acid makes cells more sensitive to the damaging effects of radiation.

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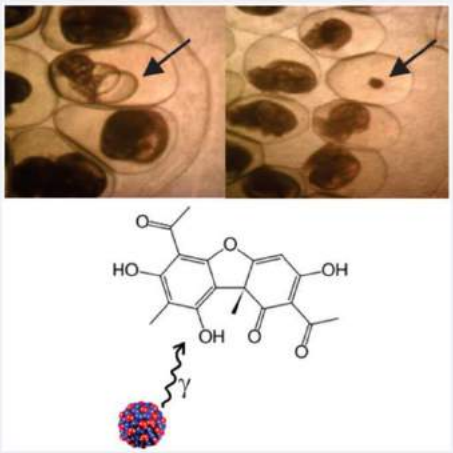
Biomphalaria glabrata; radiation; radiosensitization; usnic acid



Abbreviations: ^{60}Co: Cobalt-60; DNA: deoxyribonucleic acid; Gy: gray; LC_{50}: lethal concentration for 50%; ROS: reactive oxygen species; UA: usnic acid

Introduction

Radiosensitizers are substances that make the target cells more susceptible to the biological effects of ionizing radiation (Nambiar et al. 2011). These molecules amplify the damage caused by the interaction of radiation with DNA and other cellular structures, regardless of whether the compounds cause cell damage individually (Verma 2016). Their main application occurs in the field of oncology, where they

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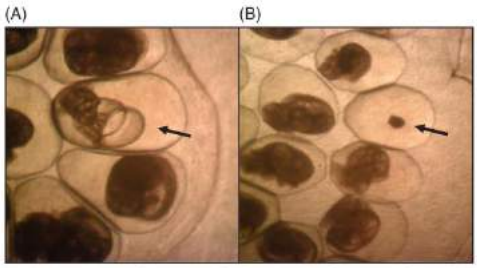

Figure 1. Spawns in veliger stage of embryonic development of *Biomphalaria. glabrata*. Viable and non-viable embryos. Indicated by black arrow: A) malformed and B) dead embryo.

are administered to enhance the effect of radiotherapy, sensitizing tumor cells and making them more susceptible to radiation damage (Javvadi et al. 2008).

These treatments also entail a risk of inducing deleterious side effects to the patients. It is, therefore, necessary to undertake studies of natural radiosensitizers, which could maintain the efficacy of the treatment with less side effects of ionizing radiation exposure, due to the possibility of reduction of the total absorbed dose. The currently used synthetic drugs have limitations due to their toxicity such as myelosuppression, hypotension, bradycardia, neuropathy, and hepatic insufficiency (Levin et al. 2000). These findings reinforce the need to study alternative substances of natural origin with radiosensitizing properties.

Among the radiosensitizers of natural origin already known are phenolic compounds and antioxidants, such as curcumin (Goel and Aggarwal 2010), found in saffron, resveratrol (Araldi et al. 2018), polyphenol (Lagerweij et al. 2016), a genotype-abundant flavonoid found in leguminous plants and quercetin (Lin et al. 2012). derived from fruits, vegetables, grains, seeds and spices.

Lichens, symbiotic associations between fungi and algae, are the source of a variety of bioactive compounds, mainly derived from their secondary metabolism and having applications in medicine, the textile industry, cosmetics and food (Kosanić et al. 2012; Manojlovic et al. 2012; Paudel et al. 2012). Usnic acid, dibenzofuran found in several species of lichens, is one of the most studied lichen metabolites and one of the few commercially available today. A number of biological activities are attributed to this metabolite, and its analgesic, antiviral, antiparasitic, antimicrobial, anti-inflammatory, antiproliferative, antitumor and antioxidant effects are reported (Shang et al. 2014; Su et al. 2014). Such characteristics make usnic acid a promising candidate for bioassays that can verify its radiosensitizing activity.

With the advancement of molecular biology and experimental embryology, evidence has confirmed the correlation between tumourigenesis and early embryonic development, so the early embryo shares many characteristics with the development of cancer, both biologically and molecularly (Williams et al. 1993; Monk 1990; Ma et al. 2010). Embryos of *Biomphalaria* sp. are considered good biological models, having a short embryoic period, simple and low cost maintenance and translucent spawnings which makes observation easy. The genus has a wide geographic distribution in tropical countries and has proved to be a useful model organism for bioassays of ecotoxicity, radioprotection and immunological tests (Oliveira-Filho et al. 2009; Siqueira et al. 2014; Sullivan and Belloir 2014). Therefore, the objective of this study is to evaluate the possible radiosensitizing effect of usnic acid using the simple biological model of *Biomphalaria glabrata* embryos.

Material and methods

Experimental animals

Pigmented adult snails of *B. glabrata* were used, measuring 10–14 mm diameter, from São Lourenço da Mata, Pernambuco and maintained for successive generations in the Radiobiology Laboratory of the Department of Biophysics and Radiobiology of Federal University of Pernambuco. The snails were kept in plastic tanks of approximately 20 L filtered and dechlorinated water, pH 7 and a temperature of about 25 ± 3 °C. The snails were fed daily with fresh organic lettuce (*Lactuca sativa*). The animals deposited their spawn onto colorless polyethylene strips (5x5 cm) that were floated on the water surface and examined under a stereomicroscope (Tecnival SQZ-SD4, São Paulo, Brazil) for individualization and identification of embryonic stage. After identification, embryos in the blastula stage were collected and divided into groups of 100 ± 3 specimens.

Determination of the lethal concentration for 50% of embryos

The toxicity of usnic acid (Sigma-Aldrich, Saint Louis, USA) in *B. glabrata* embryos was assessed in order to determine the lethal concentration for 50% (LC_{50}) as well as the concentration which showed the lowest lethality for embryos.

The assay was performed according to the method described by Oliveira-Filho and Paumgartten (2000). To perform the toxicity test, the embryos in blastula stage were placed in 24-well polystyrene plates (Prolab, São Paulo, Brazil) and exposed for 24 h to concentrations of non-irradiated and irradiated usnic acid (aqueous solution): 0.15, 0.3, 0.6, 1.2, 1.5, 2, 2.5, 5 and 10 μg/mL. For each concentration, approximately 100 embryos were used in triplicate.

After the exposure, the embryos were washed with tap water for total removal of usnic acid and then were maintained with filtered and dechlorinated water and examined under a stereomicroscope for eight consecutive days. The viability analysis was performed according to the method described by Okazaki et al. (1996). The embryos which had a normal development (during the experimental period) were considered viable and those which demonstrated malformations during development or were obviously dead were considered non-viable (Figure 1). The control group consisted of embryos maintained with filtered and dechlorinated water in the same conditions as the experimental embryos. The results were expressed as the mean percentage of non-viable embryos ± standard error of the mean (non-viable embryos %).

Table 1. Effect of usnic acid non-irradiated and irradiated with 4 Gy in embryos of *Biomphalaria glabrata*.[a]

	Concentration (µg/mL)	Non-viable embryos (%)
Control	0	1.8 ± 0.5
	0.15	1.9 ± 0.6
	0.3	2.7 ± 0.9
	0.6	4 ± 1.7
	1.2	19.3 ± 0.7*
Usnic acid	1.5	54.4 ± 3.4*
	2	98 ± 0.2*
	2.5	99 ± 0.1*
	5	99 ± 1*
	10	99.5 ± 0.5*
	0.15	1.4 ± 0.9
	0.3	3.1 ± 0.4
	0.6	3.6 ± 1.1
	1.2	23.7 ± 0.5*
Usnic acid irradiated	1.5	49.7 ± 4.8*
	2	97.3 ± 0.7*
	2.5	98.8 ± 0.5*
	5	99.2 ± 0.3*
	10	99.8 ± 0.2*

[a]The results are expressed as mean ± standard error mean. The significant differences were related between the groups by * vs. control ($p < .05$).

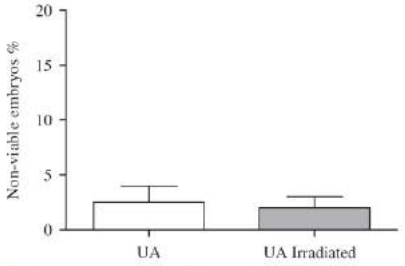

Figure 2. Embryotoxicity of usnic acid irradiated in *Biomphalaria glabrata*. The results are expressed as a mean and error bars indicate the standard deviation for $n = 30$ independent experiments. UA: embryos exposed to non-irradiated usnic acid; UA irradiated: embryos exposed to irradiated usnic acid.

Evaluation of radiosensitivity

The evaluation of the radiosensitizer capacity of usnic acid was performed with the concentration that showed the lowest toxicity (0.6 µg/mL), where the viability of the embryos was analyzed as described above. The embryos were divided into the following groups:

- Control (C): embryos maintained with filtered and dechlorinated water throughout the experimental period (eight days);
- Irradiated control (I): irradiated embryos (4 Gy) were maintained with filtered and dechlorinated water throughout the experiment;
- Usnic acid (UA): embryos exposed to usnic acid. After 24 h of exposure to the substance, the embryos were removed and kept in filtered and dechlorinated water until the end of the experiment;
- UA + bI: embryos exposed to usnic acid before being exposed to ionizing radiation. After 24 h of exposure to the substance, the embryos were removed and kept in filtered and dechlorinated water until the end of the experiment;
- UA + aI: embryos exposed to usnic acid after being exposed to ionizing radiation and after 24 h, the embryos were removed and kept in filtered and dechlorinated water until the end of the experiment.

The embryos were irradiated using a source of Gamma Cell ^{60}Co (Radionics Labs, dose rate = 2.906 Gy/h).

Statistical analysis

The results were expressed as mean ± standard error mean. The determination of the LC_{50} was analyzed by Probit regression using the software StatPlus® 2009 Professional. The difference between two groups was analyzed using a t-test, whereas the difference between three or more groups was analyzed using the analysis of variance (ANOVA) followed by Newman-Keuls test. The differences were considered significant when $p < .05$.

Results

Analysis of toxicity to embryos

The result of embryotoxicity of usnic acid is shown in the Table 1. The tested concentrations ranged from 0.15 to 10 µg/mL. The concentration of 0.6 µg/mL did not produce any change in the viability of the embryos, but for concentrations of 1.2, 1.5 and 2 µg/mL, there was an increase in the embryonic deterioration compared to the control group (19.3 ± 0.7, 54.4 ± 2.3 and 98 ± 0.2 vs. 1.8 ± 0.5). The concentration of 2 µg/mL showed a maximal response. In addition, there was no significant difference between the irradiated and non-irradiated groups. With these results, the LC_{50} of usnic acid non-irradiated was calculated, where its value was approximately 1.36 µg/mL, which is a concentration similar to that observed by Araújo et al. (2018). The LC_{50} of usnic acid irradiated was calculated, where its value was approximately 1.43 µg/mL. The highest concentration tested that did not show toxicity to embryos was 0.6 µg/mL. This concentration was selected to assess the capacity of usnic acid as a radiosensitizer.

Stability of usnic acid after irradiation

An evaluation was performed after the irradiation of the substance at a dose of 4 Gy, to verify whether there was any change in the biological activity, as measured by the embryo toxicity test with irradiated and non-irradiated solutions of usnic acid at the selected concentration of 0.6 µg/mL (Figure 2). No difference was found in the numbers of non-viable embryos in the group exposed to the irradiated substance compared to the group with the non-irradiated substance.

Radiosensitizer activity

The results of the radiosensitizer capacity of usnic acid are present in Figure 3.

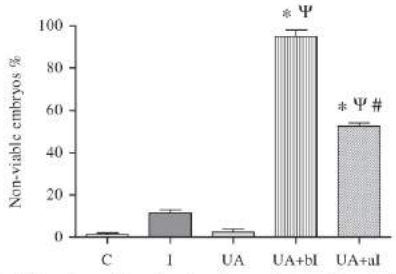

Figure 3. Effect radiosensitizer of usnic acid on embryos of *Biomphalaria glabrata*. C: control group; I: irradiated animals; UA: exposed to usnic acid; UA + bI: exposed to usnic acid before irradiation and UA + aI: animals exposed to usnic acid after irradiation. The results are expressed as a mean and error bars indicate the standard error mean for $n = 30$ independent experiments. The significant differences were related between the groups by # vs. UA + bI, Ψ vs. UA and * vs. I ($p < .05$).

The group I presented the highest number of non-viable embryos (11.5 ± 1.5) compared to group C (1.3 ± 0.9).

With regard to the usnic acid, the embryos exposed to the substance before irradiation (UA + bI) showed an increase in the number of non-viable embryos (95 ± 3) when compared to groups UA (2.5 ± 1.5) and I (11.5 ± 1.5).

Those embryos that were exposed to usnic acid after irradiation (group UA + aI) also showed an increase in the number of non-viable embryos (52.5 ± 1.5) in comparison to the groups UA (2.5 ± 1.5) and I (11.5 ± 1.5), however, when compared to animals that were exposed to group UA + bI (95 ± 3), the later presented a higher number of non-viable embryos.

Discussion

In recent years, lichens have been widely studied for their potential pharmacological properties. Their metabolites have also received special attention due to antibacterial (Segatore et al. 2012), antifungal (Shahi et al. 2011), antioxidant (Ranković et al., 2010) and anti-tumor properties (Einarsdóttir et al. 2010). In the field of radioprotection, great emphasis has been given to the use of natural products as radioprotectors or radiosensitizers as these may have advantages over synthetic compounds in, for example, producing fewer unwanted side effects.

The results of this study demonstrated an increase in mortality of embryos exposed to usnic acid in low concentrations, possibly due to the harmful effects caused by reactive oxygen species and mitochondrial dysfunction, both of which may be related to the induction of apoptotic mechanisms in embryonic cells. These effects were corroborated by studies in human liver cells (Chen et al. 2015) and rats (Han et al. 2004). In tests with neural cells exposed to usnic acid, there was evidence of a cytotoxic effect expressed as morphological changes and reduced viability which may lead to cell death possibly due to the increased production of reactive oxygen species (ROS; Rabelo et al. 2012). In other organisms similar toxicity was observed as demonstrated by Luz et al. (2015), who evaluated the action of usnic acid for promastigotes of *Leishmania infantum chagasi*. It was verified that the substance presented a leishmanicidal effect possibly related to morphological alterations in parasite cells. In addition, similar results were obtained in studies performed to verify the activity of usnic acid and its derivatives against the influenza A virus (H1N1)2009, where the lichen-derived substance showed antiviral activity (Sokolov et al. 2012).

The embryo toxicity test was first used in order to check whether 4 Gy irradiation might cause changes in the biological activity of usnic acid. No significant differences in the number of non-viable embryos were found, 4 Gy is a low dose in terms of changing chemical compounds (but not in terms of biological effects on DNA and other biomolecules) when compared with the doses used in the literature (Okazaki et al. 1996).

However, when the radiomodifying activity was investigated, it was demonstrated that the exposure of *B. glabrata* embryos to usnic acid, both before and after irradiation, the animals became more susceptible to the damaging effects of ionizing radiation. It has been suggested that this enhanced toxicity could be related to the increase of ROS present in the extra and intracellular environment. Possibly, usnic acid, when utilized in combination with ionizing radiation, may inhibit or reduce the activity of antioxidant enzymes or the molecular mechanisms of DNA repair or even the proliferative state of the cells which make them more susceptible to suffer biological damage.

Until now no studies have been reported evaluating the radiosensitizing capacity of usnic acid. However, some studies have demonstrated an antiproliferative activity of this substance (Bessadottir et al. 2012; Singh et al. 2013), which are directly related to the characteristic of a radiomodifier, more specifically, a radiosensitizer (Grem 2000). Other studies performed with cell lines of breast carcinoma (T-47D cell line human) and human pancreatic cancer (Capan-2), have demonstrated that usnic acid showed antiproliferative activity at a concentration of 4 μg/mL against the T-47D cell line and 5 μg/mL against Capan-2 cells (Einarsdóttir et al. 2010).

El-Beltagi et al. (2011) assessed the interaction of gamma radiation on the production of secondary metabolites in rosemary (*Rosmarinus officinalis* L.). They found that ionizing radiation induced an increase in the concentrations of phenols, flavonoids, amino acids, proteins and sugars. Similar results were obtained by Hussain et al. (2013), who studied the effect of ionizing radiation on damask and observed the occurrence of increased levels of flavonoids, phenols and β-carotene. In studies using *Ginko biloba* exposed to ionizing radiation phenolic compounds present in the plant extract showed a significant increase in comparison to a control group, with a dose-dependent response (Pereira et al. 2015). These alterations in the structure of the molecule may occur, since radiation can break chemical bonds and thus modify the structural characteristic of a substance, therefore, it is capable of altering the activity of a molecule, such as, increasing or decreasing toxicity for some organisms (Siqueira et al. 2014). Thus, depending on the substance

exposed to radiation, substances can be formed with radiosensitizing or radioprotective properties.

The present work has shown the potential of usnic acid being used in combination with ionizing radiation to enhance the destruction of groups of cells. If this phenomenon were to be applied to radiotherapy lower radiation doses could be needed to achieve the same tumor control as with present treatment regimens and with consequently better sparing of healthy tissue. Similarly, tests (Katz et al. 2008) using soy isoflavone and genistein alone or in combination with gamma radiation have led to the improved inhibition of cell growth in the human salivary gland. Moreover it has also been shown that the use of certain substances in combination with radiotherapy can increase the effectiveness of treatment of head and neck cancer (Katz et al. 2008). In studies performed with curcumin, it has been shown that it exhibited a potentially radiosensitizing effect, observed by optimization of the radiotherapy, either by increasing the number of destroyed tumor cells or allowing a decrease of the dose needed to produce the same therapeutic effect, and further, to reduce and so reducing the potential side effects due to ionizing radiation (Javvadi et al. 2008). Parthenolide is another substance shown to have a radiosensitizing effect when, combined with the radiation-induced increase of ROS levels reducing thiol in cancerous prostate cells (Watson et al. 2009). Another substance that has radiosensitizer action against cancer cells was ferulic acid. When associated with ionizing radiation, it produced a decrease in the viability of cells and also a decrease of antioxidant capacity and an increase of intracellular ROS, lipid peroxidation and DNA damage in HeLa and ME-80 human cells lines (Karthikeyan et al. 2011). Similar effects were also observed in studies performed with ellagic acid, where tumor cells treated, in vitro, with the combination of the phenolic compound and exposure to ionizing radiation with 6 Gy exhibited high levels of ROS. Changes in the transmembrane potential of mitochondria, reduced levels of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, together with a decrease in cell viability 24 h after treatment, in vivo, were found when using ellagic acid together with ionizing radiation in fractionated doses of 2 Gy (Bhosle et al. 2005).

Conclusion

This study has demonstrated the radiosensitizing effect of usnic acid on *B. glabrata* embryos exposed to ionizing radiation. The mechanisms responsible for potentiation of the damage caused by the combination of radiation with usnic acid may involve the action of bioactive free radicals. Further studies are necessary, commencing perhaps with human cancer cell lines, to evaluate the applicability of the combination of this substance with radiation with a view to its potential applications in radiotherapy.

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