UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
DEPARTAMENTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

DANILO RAMOS CAVALCANTI

AVALIAÇÃO DO POTENCIAL DE COMPOSTOS NATURAIS COMO POSSÍVEL ALTERNATIVA TERAPÊUTICA CONTRA *Trichomonas vaginalis*

Recife
2018

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de Doutor em Ciências Biológicas.

Área de concentração: Biotecnologia.

Orientadora: Prof. Dra. Márcia Vanusa da Silva.
Coorientador: Prof. Dr. Antonio Pereira Neves Neto.

Recife

2018

Dados Internacionais de Catalogação na Publicação (CIP) de acordo com ISBD

Cavalcanti, Danilo Ramos
Avaliação do potencial de compostos naturais como possível alternativa terapêutica contra *Trichomonas vaginalis* / Danilo Ramos Cavalcanti. – 2018.

132 f. : il.

Orientadora: Márcia Vanusa da Silva.
Coorientadora: Antônio Pereira das Neves Neto.
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Pós-graduação em Ciências Biológicas, Recife, 2018.
Inclui referências e anexos.

1. Doenças parasitárias. 2. Farmacologia. I. Silva, Márcia Vanusa da (Orientadora). II. Neves Neto, Antônio Pereira das (Coorientador). III. Título.

616.951 CDD (22.ed.) UFPE/CB – 2018 - 257

Elaborado por Bruno Márcio Gouveia - CRB-4/1788

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Aprovada em: _____/_____/_____.

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Dedico esse trabalho a Deus, simplesmente por tudo. Aos meus pais, Manoel Candido Cavalcanti e Célia Maria Ramos Cavalcanti, por todo amor incondicional e o investimento na minha formação educacional. À minha esposa, Julyana Viegas Campos Cavalcanti, pelo companheirismo em todas as horas. À minha irmã, Diana Ramos Cavalcanti, pelo apoio e incentivo.

AGRADECIMENTOS

A Deus, porque sem Ele, nada do que fiz teria sentido para mim. Pelas vezes em que fui fortalecido quando pensava em desistir. Honra e glória sejam dadas a Ele.

Aos meus pais, Manoel e Célia, pelo amor, carinho, paciência, incentivo e determinação em me conduzirem até o presente momento. Amo vocês!

À minha irmã, Diana, pelo apoio nas horas em que preciso.

À Julyana Viegas Campos Cavalcanti, minha esposa, amiga, companheira, que sempre me incentiva, me ajuda e me apoia em minhas decisões. Te amo!

Aos meus avós, tios e tias, primos e primas, que sempre torcem por meu avanço profissional.

À minha sogra, Glauce de Almeida Viegas, pelo apoio e torcida nessa conquista.

À Prof. Dra. Márcia Vanusa da Silva, por mais uma vez ter confiado em mim para realização deste projeto.

Ao Dr. Antonio Pereira das Neves Neto, pela orientação, pelas críticas e sugestões construtivas e pelo ensino em todas as instâncias deste projeto. Obrigado pelo aceite na co-orientação. Sua participação foi crucial para a realização deste projeto.

À Mary Aranda, por ter me ensinado e me ajudado nos momentos mais difíceis durante esses 4 anos de doutorado. Dispondo-se sempre a me ajudar, mesmo nos finais de semana, indo para o laboratório e ficando até tarde. Muito obrigado!

Aos meus ICs, Tuanne e Geyson, por me ajudarem em várias etapas deste projeto.

Aos amigos dos laboratórios de Biologia Celular de Patógenos, Larissa, Taciana, Carina Helena, Vanderlan, Rosi, Rômulo, Gilsan, Ana Carla, Rogério, Rayana, Gabriela, Victor e Lays pelo apoio, risadas e altos momentos de descontração para aliviar a tensão dos experimentos.

À Dra. Regina Célia Bressan Queiroz de Figueiredo por abrir as portas de seu laboratório para mim.

Ao Instituto Aggeu Magalhães, pela infraestrutura fornecida para o desenvolvimento dessa tese.

Ao Programa de Pós-graduação em Ciências Biológicas e a todos que fizeram e/ou fazem parte da coordenação deste programa.

À CAPES pelo suporte financeiro.

Porque melhor é a sabedoria do que joias, e de tudo o que se deseja nada se pode comparar com ela.

Provérbios 8:11

RESUMO

O protista parasito *Trichomonas vaginalis* é o agente etiológico da tricomoníase, a infecção sexualmente transmissível não viral mais comum no planeta. A infecção está associada a sérias consequências para a saúde e o número de casos de resistência ao fármaco de escolha, o metronidazol, está em constante crescimento. Sendo assim, a necessidade por alternativas para o tratamento da tricomoníase torna-se evidente. O objetivo desta tese foi avaliar o potencial do extrato metanol:água de (-)-epigalocatequina-3-galato (EGCG) e *Bredemeyera floribunda* (BFRMA) como possíveis alternativas quimioterápicas contra *T. vaginalis*. Para investigar se os compostos testados apresentariam efeitos antiproliferativos, foram testadas diferentes concentrações de droga em *T. vaginalis*. Para verificar se os mesmos exerceriam efeitos citotóxicos sobre as células HeLa (uma linhagem de células de câncer de colo de útero), os ensaios foram avaliados pela técnica do brometo de 3-(4,5-dimetiltiazol)-2,5-difeniltetrazólio (MTT). Curvas de crescimento foram feitas para determinação da concentração inibitória média (CI_{50}), e com este valor foram realizados testes para verificar a alteração na morfologia dos parasitos tratados com os produtos naturais. Os resultados mostraram que o BFRMA apresentou maior atividade frente ao parasito em comparação com o EGCG em 24 horas. Ambos apresentam citotoxicidade contra células HeLa conforme o aumento da concentração testada, 100% e 99,2% para BFRMA e EGCG, nas maiores concentrações testadas. Alterações na ultraestrutura de parasitos tratados com os compostos apresentaram características típicas da morte celular, como a formação de *shedding* de membrana, intensa vacuolização no citoplasma, formação de nucleoide em hidrogenossomos, expansão de retículo endoplasmático, complexo de Golgi danificados, ruptura de membrana e lise celular. Diante disso, conclui-se que os resultados apresentados demonstraram o potencial dos produtos naturais de plantas contra *T. vaginalis* e contribuem para o entendimento das propriedades farmacológicas nas modificações na morfologia deste parasito, bem como para o desenvolvimento racional de alternativas para o tratamento da tricomoníase.

Palavras-chave: Alterações ultraestruturais. Citotoxicidade. *Trichomonas vaginalis*.

ABSTRACT

The protist parasite *Trichomonas vaginalis* is the etiologic agent of trichomoniasis, the most common non-viral sexually transmitted infection on the planet. Infection is associated with serious health consequences and the number of drug resistance cases of choice, metronidazole, is constantly growing. Therefore, the need for alternatives for the treatment of trichomoniasis becomes evident. The objective of this thesis was to evaluate the potential of the methanol: water extract of (-)-epigallocatechin-3-gallate (EGCG) and *Bredemeyera floribunda* (BFRMA) as possible chemotherapeutic alternatives against T. vaginalis. To investigate whether the tested compounds would have antiproliferative effects, different concentrations of drug were tested in *T. vaginalis*. To verify if they exert cytotoxic effects on HeLa cells (a lineage of cervical cancer cells), the assays were evaluated by the 3-(4,5-dimethylthiazole)-2,5-diphenyltetrazolium bromide technique (MTT). Growth curves were made to determine the mean inhibitory concentration (IC_{50}), and with this value tests were performed to verify the alteration in the morphology of the parasites treated with the natural products. The results showed that BFRMA showed higher activity against the parasite compared to EGCG in 24 hours. Both exhibit cytotoxicity against HeLa cells as the concentration increased, 100% and 99.2% for BFRMA and EGCG, at the highest concentrations tested. Alterations in the ultrastructure of parasites treated with the compounds showed typical characteristics of cell death, such as the formation of membrane shedding, intense vacuolization in the cytoplasm, nucleoid formation in hydrogenosomes, endoplasmic reticulum expansion, damaged Golgi complex, membrane rupture and lysis cell phone. Therefore, it is concluded that the results presented demonstrated the potential of natural plant products against T. vaginalis and contribute to the understanding of the pharmacological properties in the modifications in the morphology of this parasite, as well as the rational development of alternatives for the treatment of trichomoniasis.

Key-words: Ultrastructural changes. Cytotoxicity. *Trichomonas vaginalis*.

SUMÁRIO

1 INTRODUÇÃO

A tricomoníase é a infecção sexualmente transmissível (IST) não viral mais comum do planeta, causada pela colonização no trato urogenital feminino e masculino por meio do protista flagelado *Trichomonas vaginalis* (WHO, 2012; MARITZ et al., 2014). A Organização Mundial da Saúde (OMS) estima uma incidência de 276 milhões de novos casos por ano e uma prevalência de 187 milhões de indivíduos infectados na faixa etária dos 15 aos 49 anos de idade. A incidência dessa infecção abrange vários fatores, dentre eles: idade, atividade sexual, número de parceiros sexuais, outras ISTs, ciclo menstrual e condições socioeconômicas (WHO, 2012).

A infecção em mulheres está associada a sintomas como secreção vaginal (devido à infiltração de leucócitos polimorfonucleares no fluido vaginal), odor, prurido e irritabilidade. Além disso, pode ocasionar problemas mais graves como aborto, parto prematuro, baixo peso ao nascer e infertilidade. Aproximadamente 75% dos homens são assintomáticos, porém a tricomoníase tem sido associada como um fator de risco para o desenvolvimento de câncer de próstata por várias razões, tais como: capacidade de provocar inflamação pelo fator inibidor da migração de macrófagos (através da produção de anticorpos em indivíduos infectados, promovendo a proliferação e invasão de células da próstata e estimulando as vias celulares) e danos no epitélio da próstata, tropismo prostático e tendência a formar inflamações subclínicas e crônicas, uretrite e raramente balanite (MIELCZAREK e BLASZKOWSKA, 2016; HIRT E SHERRARD, 2015, TWU et al., 2014). Estudos in vitro associam o aumento da expressão de protooncogenes na indução do crescimento e invasão de células da próstata (TWU et al., 2014; ZHU et al., 2016; SUTCLIFFE et al., 2012).

A infecção com *T. vaginalis* está também relacionada ao aumento da suscetibilidade ao vírus da imunodeficiência humana, ao vírus do herpes e à infecção pelo papilomavírus, podendo evoluir para câncer cervical (KISSINGER, 2015; MEITES et al., 2015; SILVER et al., 2014). A conseqüência da negligência para com a doença, gera os altos custos e o fardo dos cuidados de saúde associados a tricomoníase ultrapassam 24 milhões de dólares por ano nos Estados Unidos (OWUSU-EDUSEI et al., 2013). O custo estimado das infecções por HIV-*T. vaginalis* é de aproximadamente 167 milhões de dólares por ano (CHESSON, BLANDFORD e PINKERTON, 2004).

Derivados nitromidazólicos, como o metronidazol, constituem a única fonte de tratamento contra a tricomoníase aprovada pela OMS e o Ministério da Saúde do Brasil

(MEITES, 2013; KISSINGER, 2015). Este composto vem sendo utilizado por mais de 50 anos no tratamento da doença e, apesar de apresentar um alto grau de eficácia, possui pontos negativos que devem ser levados em consideração. O medicamento provoca uma série de efeitos colaterais nos pacientes, tais como náuseas, vômitos, gosto metálico na boca, diarreia e neuropatias periféricas (PEARLMAN et al., 1996). Além disso, de maneira preocupante, diversos casos de resistência ao metronidazol vêm sendo documentados. Aproximadamente 5% dos casos de tricomoníase apresentam algum nível de resistência ao metronidazol (DAS, HUENGSBERG e SHAHMANESH, 2005; KIRKCALDY et al., 2012; SCHWEBKE e BARRIENTES, 2006). Em acréscimo, o metronidazol não pode ser administrado em mulheres grávidas até o terceiro mês de gestação devido à falta de estudos sobre possíveis efeitos teratogênicos. É também possível que, durante o tratamento da tricomoníase, o metronidazol provoque um processo inflamatório no hospedeiro devido à morte por necrose do parasito, seguido pela liberação de alguns componentes que podem ativar o sistema imunológico hospedeiro (BURTIN et al., 1995; PIPER, MITCHEL e RAY, 1993; SHEEHY et al., 2015).

Apesar do quadro acima delineado, as grandes indústrias farmacêuticas optaram por relegar a um segundo plano a pesquisa na área de desenvolvimento de novas alternativas quimio ou imunoterapêuticas contra tricomoníase. O sentimento entre muitos profissionais de saúde, gestores de saúde, população e mídia em geral é de que a tricomoníase não apresenta um grau de incidência e morbidade expressivos. Para agravar ainda mais, o Programa Nacional de IST/AIDS do Ministério da Saúde do Brasil estabeleceu um sistema de vigilância das ISTs de notificação não-compulsória e de determinadas doenças específicas consideradas de interesse nacional no qual a tricomoníase não foi incluída. Pela sua magnitude, transcendência, vulnerabilidade e factibilidade de controle, a tricomoníase deve ser priorizada enquanto agravo em saúde pública.

Portanto, as severas limitações supramencionadas e a alta incidência da tricomoníase conduzem à necessidade pela busca de compostos que sejam mais eficazes contra os parasitos e menos tóxicos para os pacientes em tratamento, desempenhando, assim, um papel fundamental na saúde pública de países em desenvolvimento como o Brasil. Cabe sempre lembrar que o Brasil, como um dos países líderes na atividade científica entre os países do mundo em desenvolvimento, tem grande responsabilidade no estudo de doenças que predominam nesses países. O estado de Pernambuco, em particular, por abrigar instituições

de vanguarda na ciência biomédica nacional, tem responsabilidade especial nesta área. Por todos estes fatores há um consenso de que o desenvolvimento de novas alternativas quimio-terapêuticas é uma prioridade.

Sendo assim, os produtos naturais surgem como alternativas promissoras para o tratamento da tricomoníase, uma vez que constituem uma fonte rica de moléculas ativas e de grande potencial. A utilização de plantas medicinais para o tratamento de diversas doenças é descrita desde milhares de anos atrás, datando de civilizações como antiga Babilônia, Egito, Índia e China. Na indústria farmacêutica moderna, os produtos naturais continuam desempenhando um papel fundamental no desenvolvimento de fármacos, mesmo com a grande variedade de moléculas derivadas da química combinatória (NGO et al., 2013).

O entendimento da variedade química dos produtos naturais fornece uma importante estratégia para o desenvolvimento racional de fármacos. Neste sentido, a grande biodiversidade coloca o Brasil em uma posição estratégica para o desenvolvimento racional e exploração sustentável de novos metabólitos com valor terapêutico. Os produtos naturais contribuem há mais de 30 anos de maneira significativa na obtenção de novos fármacos de origem natural ou de derivados sintéticos (NEWMAN e CRAGG, 2012).

Diversos estudos têm sido realizados no intuito de mostrar a eficácia de produtos naturais como possíveis drogas para tratamento da tricomoníase (VIEIRA et al., 2011; GIORDANI et al., 2011; VIEIRA et al., 2015; ROCHA et al., 2012; NEWMAN e CRAGG, 2012; MORAES et al., 2012). Entretanto, até o presente momento nenhuma droga foi lançada na indústria farmacêutica como alternativa contra cepas de *T. vaginalis* resistentes ao metronidazol.

Sendo assim, os objetivos do presente estudo foram avaliar o potencial de produtos naturais de plantas como possíveis alternativas quimioterápicas contra *T. vaginalis*; realizar uma revisão da literatura mostrando a atividades de produtos naturais com atividade contra *T. vaginalis*; avaliar a atividade anti-*T. vaginalis* do extrato metanol:água de raízes de *Bredemeyera floribunda* e da epigalocatequina-3-galato (EGCG); analisar as alterações ultraestruturais provocadas pelos produtos naturais na morfologia de *T. vaginalis* e avaliar a citotoxicidade dos produtos naturais frente a células HeLa.

2 REVISÃO DA LITERATURA

2.1 CLASSIFICAÇÃO TAXONÔMICA

Trichomonas vaginalis (DONNÉ, 1836) é um protozoário parasito pertencente ao Filo Parabasalia (CEPICKA et al., 2010; NODA et al., 2012). Este filo consiste em um grupo de protistas flagelados desprovidos de mitocôndrias que se caracterizam pela presença de organelas de origem endossimbiótica relacionadas às mitocôndrias, denominadas hidrogenossomos (LINDMARK e MÜLLER, 1973), de um aparato parabasal (complexo de Golgi associado com filamentos estriados chamados de filamentos parabasais) (HONIGBERG e BRUGEROLLE, 1990), do complexo pelta/axóstilo (sistema de microtúbulos) (BENCHIMOL et al*.,* 1993) e por apresentarem uma mitose fechada com fuso extranuclear (criptopleuromitose). Além disso, estes protistas não apresentam peroxissomos e possuem ribossomo híbrido 80S composto por um conjunto completo de proteínas eucarióticas e RNA ribossomais 16S e 23S parecidos com os de procaritos (LI et al., 2017).

O filo Parabasalia abrange diversos protistas importantes tanto na área médica quanto ecológica, incluindo também a maioria dos simbiontes intestinais flagelados de térmitas (BRUNE e DIETRICH, 2015) e parasitas humanos (HIRT e SHERRARD 2015; KUSDIAN e GOULD, 2014). Dentre os representantes mais conhecidos e estudados deste grupo encontram-se os patógenos, *Tritrichomonas foetus* (RIEDMÜLLER, 1928), parasito do trato urogenital bovino e do trato intestinal de felinos, suínos e cães, *Tetratrichomonas gallinarum* (MARTIN e ROBERTSON, 1911), parasito da cavidade bucal e do ceco de aves e *Tritrichomonas muris* (GRASSÉ, 1926).

Embora ainda não esteja claro o grau de parentesco entre as diversas espécies do filo Parabasalia, estudos filogenéticos apontam que esses organismos encontram-se entre os mais primitivos eucariotos (NODA et al., 2012).

A classificação atual de *T. vaginalis* foi proposta por HAMPL et al. (2006)

Filo Parabasalia

Classe Trichomonadea

Ordem Trichomonadida

Família Trichomonadidae

Gênero *Trichomonas*

Espécie *Trichomonas vaginalis*

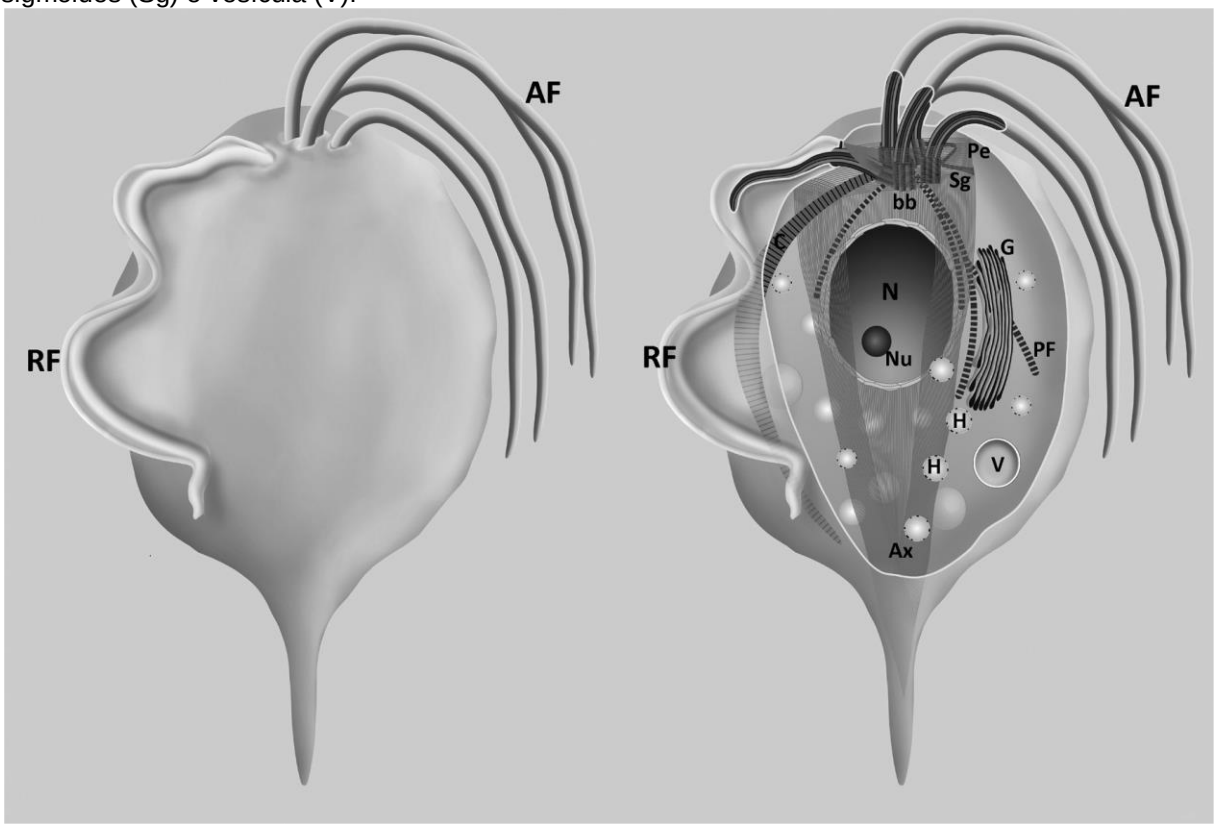
2.2 BIOLOGIA DE *T. vaginalis*

T. vaginalis é um parasito extracelular, eucarionte e unicelular, que se reproduz por divisão binária (KIRBY, 1951; MATTOS et al., 1997). *T. vaginalis* possui quatro flagelos anteriores de tamanho aproximadamente semelhante (cerca de 13 µm) e outro recorrente que se destaca do corpo celular antes da região posterior final do corpo do parasito. Além do formato piriforme, que é o mais comum, em meios de cultura axênicos, este parasito pode ainda assumir duas outras morfologias: a ameboide (mais frequente em interações com a células-alvo) e o pseudocisto (que surge em situações de estresse quando o parasito internaliza seus flagelos). Formas císticas verdadeiras não são encontradas em *T. vaginalis*. (BENCHIMOL, 2004).

Um fato bastante conhecido é a mudança drástica de forma que o parasito assume após a adesão, tornando-se ameboide, sendo este considerado um sinal de virulência (ARROYO et al., 1993). Este mecanismo pode estar relacionado a uma maior superfície de adesão entre as duas células, estando as duas membranas em tão íntimo contato que parecem estar em continuidade em alguns pontos (FURTADO e BENCHIMOL, 1998).

O citoesqueleto de *T. vaginalis* (Figura 1) consiste em várias estruturas características que incluem a pelta, o axóstilo e a costa (CEPICKA et al., 2010). O sistema cariomastigonte compreende os corpos basais, o centro organizador de microtúbulos, os flagelos e os filamentos que os conectam ao núcleo (BRUGEROLLE, 1991). O axóstilo constitui-se como uma folha de microtúbulos que forma um tubo oco, o qual é aberto na região anterior formando uma área similar a uma colher, chamada capitulum. A pelta corresponde a uma fita crescente dos microtúbulos na face interna do capitulum, que se estende para circundar apicalmente as paredes do canal periflagelar. A costa apresenta estrutura composta principalmente de proteínas e sua função está relacionada ao suporte mecânico para a membrana ondulante (ROSA et al., 2013).

Figura 1 – Representação esquemática tridimensional de *T. vaginalis* mostrando a visualização externa (esquerda) e as estruturas celulares internas (direita). Destaque para o flagelo recorrente (RF), quatro flagelos anteriores (AF), axóstilo (Ax), corpos basais (bb), costa (C), complexo de Golgi (G), hidrogenossomos (H), pelta (P), núcleo (N), nucléolo (N), filamentos parabasais (PF), filamentos sigmoides (Sg) e vesícula (V).



Fonte: Benchimol, Pereira-Neves e Souza (2016).

2.3 ASPECTOS MORFOLÓGICOS DE *T. vaginalis*

2.3.1 Superfície Celular

A superfície celular de *Trichomonas* possui grande interesse em pesquisas moleculares de interação parasito-célula hospededeira, uma vez que é o ponto de contato com a célula hospedeira. Diversos carboidratos, incluindo ácido siálico, manose, galactose, N-acetilglicosamina e N-acetil-galactosamina foram identificados na superfície deste parasito, bem como nas membranas de compartimentos intracelulares (BENCHIMOL et al., 1981b, 1982b; BENCHIMOL e BERNARDINO, 2002). Receptores para moléculas carreadoras de íons ferro tais como transferrina e lactoferrina, foram identificados na membrana do parasito (SUTAK et al., 2008), assim como, receptores de superfície capazes de reconhecer proteínas de matriz extracelular, como fibronectina e laminina-1 (SILVA-FILHO e DE SOUZA, 1988; PETRÓPOLIS et al., 2008).

Em experimentos utilizando a linhagem celular MDCK (Madin-Darby Caninespaniel Kidney) (SILVA-FILHO e DE SOUZA, 1988) ou HeLa (PETRÓPOLIS et al., 2008), foi observado que a adesão do parasito é intensificada pela laminina. *Trichomonas* interage com o ambiente vaginal, utilizando neste processo moléculas envolvidas com a transdução de sinais, adesão à célula epitelial, interação com a matriz extracelular e com a citotoxicidade (SILVA-FILHO e DE SOUZA, 1988; SILVA-FILHO et al., 1989; SILVA-FILHO e BONILHA, 1992; DE JESUS et al., 2006). No ambiente onde o parasito se encontra ocorrem intensas mudanças hormonais, o que é de grande importância, pois há indícios do aumento da adesão de tricomonas em interação com células MDCK sob ação do estrogênio (SILVA-FILHO e BONILHA, 1992).

2.3.2 Hidrogenossomos

Espécies de Trichomonadideae apresentam organelas de grande importância relacionadas às mitocôndrias: os hidrogenossomos. O termo hidrogenossomo foi proposto por LINDMARK e MÜLLER (1973) devido à atividade produtora de hidrogênio molecular da organela que oxida o piruvato ou o malato em ácidos orgânicos, resultando na síntese de ATP, essencial para o metabolismo energético (MÜLLER, 1993).

Em *T. vaginalis*, os hidrogenossomos são organelas esféricas com dimensões de 0,7 x 0,5 µm, que se apresentam dispostos preferencialmente próximos ao axóstilo e a costa. As inclusões intrahidrogenosomais foram evidentes como corpos densos de elétrons localizados em uma extremidade dos perfis organelas elipsoidais, apenas dentro da membrana que limita a organela (CHAPMAN et al., 1985). Por meio da técnica de microanálise por raios-X, identificou-se a presença de íons divalentes, tais como os de cálcio, magnésio e zinco, além da presença de cobalto e alumínio (RIBEIRO et al*.,* 2001), além de alto nível de fósforo, que provavelmente se encontra sob a forma de pirofosfatos (CHAPMAN et al., 1985).

Várias similaridades foram observadas entre os hidrogenossomos e as mitocôndrias, tanto em nível bioquímico quanto estrutural, sugerindo um grau de proximidade evolutiva entre as duas organelas. As principais semelhanças encontradas foram a produção de ATP pela degradação do piruvato (LINDMARK e MÜLLER, 1973); a dupla membrana (BENCHIMOL e DE SOUZA, 1983); o mecanismo de biogênese, que ocorre por segmentação e partição (BENCHIMOL et al*.,* 1996a; 1996b); o sequestro de íons cálcio (RIBEIRO et al., 2001); as

enzimas envolvidas na formação de centros de ferro-enxofre (CARLTON et al., 2007; SCHNEIDER et al., 2011); a cardiolipina, um lipídeo encontrado na membrana mitocondrial interna e na membrana plasmática de bactérias (DE ANDRADE ROSA et al., 2006); a NADH-desidrogenase, uma enzima constituinte da cadeia transportadora de elétrons mitocondrial (CARLTON et al., 2007); e participação no metabolismo de aminoácidos (CARLTON et al., 2007; SCHNEIDER et al., 2011).

Além disso, verificou-se que os hidrogenossomos e mitocôndrias possuem mecanismos de importação de proteínas similares (DYALL et al., 2000; CARLTON et al., 2007; RADA et al., 2011). Várias chaperonas (Hsp70, Hsp60, Hsp10) com sequências sinais similares às sequências de importação para mitocôndrias foram identificadas durante a translocação de proteínas para os hidrogenossomos (BUI, BRADLEY e JOHNSON, 1996; RADA et al., 2011; SCHNEIDER et al., 2011).

Entretanto, existem algumas diferenças entre ambas as organelas. Os hidrogenossomos não possuem citocromos, enzimas do ciclo de Krebs, atividade de F0F1 ATPase, material genético e ribossomos (LLOYD et al., 1979; TURNER e MÜLLER, 1983; CLEMENS e JOHNSON, 2000). Todavia, os hidrogenossomos possuem enzimas que as mitocôndrias não apresentam, como a Fe-hidrogenase (BUI e JOHNSON, 1996) e a piruvato-ferredoxina óxido-redutase (HRDÝ e MÜLLER, 1995).

Diversas hipóteses foram levantadas com o objetivo de se estabelecer um grau de parentesco evolutivo entre mitocôndrias e hidrogenossomos. A versão mais atual é dos hidrogenossomos terem evoluído a partir de uma origem mitocondrial (EMBLEY, 2006; HJORT et al., 2010; RADA et al., 2011; SCHNEIDER et al., 2011).

2.3.3 Retículo endoplasmático

O retículo endoplasmático (RE) de *T. vaginalis* é normalmente visto ao redor do núcleo e também formando a membrana externa do envelope nuclear, assim como nas células de eucariotos superiores (QUEIROZ et al., 1991; BENCHIMOL, 2004). Pode ser encontrado em outros locais do citoplasma, como próximo aos hidrogenossomos, podendo estar relacionado à formação das vesículas periféricas (BENCHIMOL e DE SOUZA, 1985; BENCHIMOL et al., 1996b; BENCHIMOL et al., 2000; BENCHIMOL, 2008). O RE também é observado próximo ao

complexo de Golgi, sugerindo uma comunicação entre ambas as organelas (BENCHIMOL e DE SOUZA, 1985; BENCHIMOL et al., 2001).

A vesiculação do RE durante a divisão celular, fenômeno comumente observado em células de eucariotos superiores, não foi constatada em tricomonadídeos. Sabe-se, porém, que, em *Trichomonas*, algumas cisternas do retículo se alinham de modo paralelo com os microtúbulos do fuso mitótico, o que poderia apresentar um recurso de doação ou sequestro de cálcio durante a divisão do parasito (RIBEIRO et al., 2002b).

2.3.4 Complexo de Golgi e filamentos parabasais

Nos tricomonadídeos, o aparelho parabasal é formado pelas cisternas do complexo de Golgi e pelos filamentos parabasais (BENCHIMOL, 2004). O aparelho parabasal fica localizado na região dorsal e à direita do núcleo. *T. vaginalis* possui um único Golgi muito desenvolvido, podendo alcançar cerca de 6 µm de comprimento, apresentando de 8 a 12 cisternas (BENCHIMOL et al., 2001). Ao contrário do que acontece nas células de eucariotos superiores, onde a vesiculação é observada durante a divisão celular, nos tricomonadídeos a organela permanece íntegra, apenas aumentando de tamanho por crescimento lateral e se dividindo em duas, num processo denominado golgicinese (BENCHIMOL et al., 2001).

Os filamentos parabasais são estruturas cilíndricas e periódicas, formadas por bandas claras e escuras, morfologicamente similares à costa, embora sejam mais delgados que estas. Apresentam-se em número de três, sendo denominados filamentos parabasais 1, 2 e 3 (PF1, PF2 e PF3). O PF1 tem sua origem entre os corpúsculos basais do segundo e terceiro flagelos anteriores; o PF2, entre o terceiro flagelo anterior e o recorrente; e o PF3 está localizado bem próximo ao filamento adjacente, aparecendo paralelamente ao PF1 e PF2 (LEE et al., 2009). Acredita-se que os filamentos parabasais deem suporte e mantenham o posicionamento do complexo de Golgi (HONIGBERG et al., 1971; BRUGEROLLE e VISCOGLIOSI, 1994; BENCHIMOL et al., 2001).

2.3.5 Vesículas e lisossomos

O citoplasma de *T. vaginalis* possui várias vesículas de tamanhos distintos, que compõem o sistema endocítico da célula (BENCHIMOL et al., 1990). As principais enzimas constituintes dos lisossomos de *Trichomonas* seriam hidrolases ácidas e neutras (QUEIROZ

et al., 1991). Portanto, a acidificação dos lisossomos sugeriria a presença de uma bomba de prótons nas membranas desta organela, tal como observado em eucariotos superiores. Os lisossomos têm também uma participação ativa nos processos em que se observou autofagia, tal como descrito em hidrogenossomos (BENCHIMOL, 1999).

Ao longo da infecção *T. vaginalis* pode fagocitar e endocitar moléculas e células, tais como: partículas, espermatozoides, fungos, células humanas, leucócitos hemácias e bactérias (VAZQUEZ-CARRILLO et al., 2011; MIDLEJ e BENCHIMOL, 2010; BENCHIMOL et al., 2008; PEREIRA-NEVES e BENCHIMOL, 2007; LEHKER et al., 1990 ; GONZÁLEZ-ROBLES et al., 1995 ; RENDÓN-MALDONADO et al., 1998; STREET et al., 1984; JULIANO et al., 1991; BENCHIMOL e DE SOUZA, 1995).

2.3.6 Citoesqueleto

Dentre os principais componentes do citoesqueleto dos tricomonadídeos destacam-se: o complexo pelta-axóstilo, formado por microtúbulos estáveis (BENCHIMOL, 2005; RIBEIRO et al., 2002a; 2002b; 2000); os flagelos que no caso de *T. vaginalis* são quatro anteriores e um recorrente formando a membrana ondulante no contato com a membrana plasmática (HONIGBERG et al., 1971); os corpúsculos basais e seus filamentos associados, de onde emergem os flagelos e as demais estruturas relacionadas ao movimento do parasito (HONIGBERG et al., 1971; RIBEIRO et al., 2000); a costa, estrutura protéica estriada com função de diminuir as tensões provocadas pelos batimentos do flagelo recorrente (HONIGBERG et al., 1971; BENCHIMOL, 2005) e os filamentos parabasais associados ao complexo de Golgi (HONIGBERG et al., 1971; BRUGEROLLE e VISCOGLIOSI, 1994; BENCHIMOL et al., 2001). Em *T. vaginalis* pode-se também encontrar microtúbulos lábeis como os que formam o fuso mitótico (RIBEIRO et al., 2000; 2002a).

Estudos bioquímicos, moleculares e estruturais também constataram a presença de microfilamentos de actina no citoesqueleto de várias espécies de tricomonadídeos (BRUGEROLLE et al., 1996; BRICHEUX e BRUGEROLLE, 1997; BRICHEUX et al., 1998; 2000; PEREIRA-NEVES e BENCHIMOL, 2007). Estes microfilamentos são, preferencialmente, encontrados na região cortical e nos pseudópodes das *Trichomonas* quando realizam fagocitose ou quando em adesão ao substrato ou às células-alvo (BRUGEROLLE et al., 1996; PEREIRA-NEVES e BENCHIMOL, 2007).

Estudos sobre o citoesqueleto de tricomonadídeos mostraram a modificação da morfologia do protozoário durante a atividade fagocítica ou em interação com a célula hospedeira (BRUGEROLLE et al., 1996; PEREIRA-NEVES e BENCHIMOL, 2007). Desse modo, foi possível constatar uma reorganização do citoesqueleto (principalmente dos microfilamentos de actina), já que os parasitos apresentam uma forma oval em meio axênico e se transformam em amebóides quando ingerem alguma partícula ou interagem com as células-alvo (BRUGEROLLE et al., 1996; PEREIRA-NEVES e BENCHIMOL, 2007).

2.4 INTERAÇÃO PARASITO-HOSPEDEIRO

Mesmo com a alta incidência de parasitismo de *Trichomonas vaginalis*, ainda são pouco esclarecidos os mecanismos das interações parasito-hospedeiro. *T. vaginalis* é considerado um parasito não-invasivo, extracelular, visto que não penetra no epitélio, estabelecendo uma forte relação de superfície com a célula-hospedeira. Geralmente, interdigitações são formadas quando o parasito se adere às células epiteliais (GONZÁLES-ROBLES et al., 1995; RASMUSSEN et al., 1986).

Para que se instale a tricomoníase, o parasito deve inicialmente ultrapassar a barreira de muco para interagir com as células epitéliais vaginais (ALDERETE e GARZA, 1988). Isso parece acontecer devido ao movimento dos flagelos e à ação de enzimas proteolíticas, chamadas mucinases, que digerem o muco (LEHKER e SWEENEY, 1999). Pesquisas apontam que os danos provocados pelo parasito ocorrem através de um processo dependente do contato com a célula hospedeira, sendo este um pré-requisito para a citotoxicidade (GONZÁLES-ROBLES et al., 1995; BURGESS et al., 1990; SINGH, LUCAS e FICHOROVA, 2007). Entretanto, alguns autores acreditam que proteases e outros produtos metabólicos secretados pelas tricomonas seriam responsáveis pelos efeitos citotóxicos, não havendo necessidade, portanto, do contato do parasito com as células hospedeiras (GARBER e LEMCHUK-FAVEL, 1989).

Foi proposta que parte da adesão de *T. vaginalis* às células epiteliais seria mediada por cinco grupos de proteínas denominadas de adesinas (APs) (AP65, AP51, AP33 e AP23). Demonstrou-se que as adesinas de *Trichomonas* também são capazes de se ligar a diferentes tipos celulares, como eritrócitos de diferentes espécies, *Mycoplasma hominis* e linhagens celulares como células Vero, CHO e HeLa (ADDIS et al., 2000). Isso mostrou que as adesinas

não poderiam ser consideradas ligantes exclusivos do processo de adesão nas células epiteliais.

As cisteíno-proteases constituem uma outra classe de moléculas que estaria diretamente ligada com o processo de adesão (ARROYO e ALDERETE, 1989). Uma cisteíno-protease de 30 kDa (CP-30) foi encontrada mediando processos citotóxicos de *T. vaginalis* em células HeLa e epiteliais vaginais humanas (MENDONZA-LOPES et al*.,* 2000). A CP-30 está envolvida na degradação de proteínas da matriz extracelular, como a laminina e a fibronectina, potencializando o processo de citotoxicidade do parasito (SILVA-FILHO e DE SOUZA, 1988; BENCHIMOL et al., 1990; CROUCH e ALDERETE, 1999). Desta forma, as *Trichomonas* adotariam um caráter mais invasivo, pois o acesso do parasito às camadas epiteliais profundas ocasionaria a esfoliação das camadas mais superficiais. Ruptura do epitélio e de suas junções, atingindo a lâmina basal e causando infecções múltiplas ainda podem ocorrer, dependendo da virulência da cepa (KRIEGER et al., 1985; GAULT et al., 1995). Neste ambiente há uma baixa tensão de oxigênio conferindo sucesso à infecção visto que o parasito é microaerófilo.

Recentemente, tetraspaninas, uma classe de proteínas transmembranas, foram identificadas em *T. vaginalis* e reconhecidas como fatores envolvidos na adesão às células hospedeiras e proteínas da matriz extracelular (DE MIGUEL et al., 2012; COCERES et al., 2015).

Lipofosfoglicano de *T. vaginalis* (TvLPG) medeia a adesão do parasita às células do epitélio vaginal e induz a resposta inflamatória do hospedeiro (FIGUEROA-ANGULO et al., 2012). TvLPG desencadeia a secreção de IL-8 de leucócitos, induz a produção de IgG e IgA específicos e provoca a síntese das citocinas, leucotrienos, RNI e MIP-3α (MIELCZAREK e BLASZKOWSKA, 2016; FICHOROVA, 2009) relacionadas a células Th1. O TvLPG é um dos antígenos mais comumente reconhecidos por amostras de soro de mulheres infectadas por *T. vaginalis* (BASTIDA-CORCUERA et al., 2013). Estudos mostraram níveis mais elevados de IgG específica de TvLPG em amostras de soro e secreções vaginais de mulheres infectadas por *T. vaginalis* em comparação com indivíduos de controle não infectados (MENEZES e TASCA, 2016; BASTIDA-CORCUERA et al., 2013).

Outros fatores estão associados com a citoaderência de *T. vaginalis* nas células do hospedeiro, tais como AP65 (GARCIA e ALDERETE, 2007), piruvato ferredoxina oxidoredutase (SONG, 2016), legumain-1 (RENDON-GANDARILLA et al., 2016; RESÉNDIZ-CARDIEL, ARROYO e ORTEGA-LÓPEZ, 2017), CP62, atuando também como um fator de

virulência (FIGUEROA-ANGULO et al., 2012), CP30 (MALLA et al., 2014) e CP39, encontrado nas secreções vaginais, exibe atividades citotóxicas e propriedades imunogênicas em pacientes infectados (HERNANDEZ-GUTIERREZ et al., 2004).

2.5 TRICOMONÍASE

Trichomonas vaginalis é o agente etiológico da tricomoníase, infecção sexualmente transmissível não-viral mais comum do planeta. Estima-se que 276 milhões de pessoas estão infectadas com este parasito no mundo inteiro, sendo que 90% das pessoas infectadas vivem em ambientes com recursos limitados (WHO, 2012).

Nas mulheres, o principal sítio de infecção é a vagina; por apresentar epitélio escamoso, as células dessa microbiota são preferencialmente infectadas pelo parasita, embora uretra e endocérvix sejam também alcançadas pelos trofozoítos (LEHKER e ALDERETE, 2000; MUZNY e SCHWEBKE, 2013; SPARKS, 1991). O pH vaginal normal é de 4,5, sendo aumentado para 5 ou mais na presença de *T. vaginalis*. Este aumento no pH diminui a quantidade de *Lactobacillus acidophilus*, células da microbiota que protegem o epitélio e, consequentemente, torna o ambiente favorável para a multiplicação de bactérias anaeróbicas responsáveis pela vaginose bacteriana (PETRIN et al., 1998; HIRT e SHERRARD, 2015).

Entre as mulheres sintomáticas, as principais queixas são corrimento vaginal, prurido, odor e irritação (MUZNY e SCHWEBKE, 2013). A secreção vaginal acontece devido à infiltração leucocítica intensa dentro do trato genital em consequência da morte de células epiteliais, promovendo a inflamação e levando a um aumento do número de leucócitos polimorfonucleares no líquido vaginal (LAZENBY, SOPER e NOLTE, 2013). Este corrimento apresenta-se de forma espumosa, com coloração amarelada/esverdeada; porém, o aspecto e a consistência do mesmo podem ser variáveis entre as pacientes (SWYGARD et al., 2004; SCHWEBKE e BURGESS, 2004). Além disso, mulheres com tricomoníase podem apresentar a vagina e o colo do útero eritematosos e edematosos, com manchas hemorrágicas na mucosa, conhecidas como *colpitis macularis*; outras relatam disúria e dores abdominais.

Os sintomas são cíclicos e mais intensos no perÍodo mestrual por causa do efeito do ferro sobre a biologia do parasito (PETRIN et al., 1998). *T. vaginalis* pode fagocitar hemácias para a aquisição de ferro da hemoglobina, bem como para obtenção de ácidos graxos, já que o parasito é incapaz de sintetizar lipídeos (LEHKER e ALDERETE, 1991). O

muco cervical é deficiente em complemento e o sangue menstrual representa a única fonte de complemento na vagina. Uma vez que o número de organismos na vagina diminui durante a menstruação, os fatores de virulência mediados pelo ferro contribuem para a exacerbação dos sintomas neste período (PETRIN et al., 1998)

Relatos clínicos como vaginite, cervicite, endometriose e inflamação pélvica atípica são distúrbios do trato genital feminino que estão associados à infecção por *T. vaginalis* (FICHOROVA, 2009; CHERPES et al., 2006). Além destes, distúrbios na gravidez, baixo peso ao nascer, ruptura prematura das membranas e nascimento pré-termo também são consequência desta infecção (SILVER et al., 2014, COTCH et al., 1997). Estudos mostraram que o parasita aumenta 1,9 vezes o risco de câncer cervical (ZHANG e BEGG, 1994; VIIKKI et al., 2000).

A natureza oxidativa dos fluidos genitais, bem como a alta concentração de zinco no fluido prostático atuam como fatores tricomonicidas. No entanto, esta infecção causa uretrite, secreção mucopurulenta, disúria e prurido leve ou sensação de queimação imediatamente após o sexo (PETRIN et al., 1998). Outras complicações incluem prostatite, balanopostite, epididimite e, possivelmente, infertilidade (GIMENES et al., 2014). *T. vaginalis* também pode estar relacionado ao câncer de próstata em homens (TWU et al., 2014; STARK et al., 2009; SUTCLIFFE et al., 2006; SUTCLIFFE et al., 2009).

A inflamação induzida por fator de migração de macrófagos e a proliferação celular podem contribuir para a promoção e progressão do câncer de próstata. Demonstrou-se que o fator de migração de macrófagos induzidos por *T. vaginalis* provoca a produção de anticorpos em indivíduos infectados, promove a proliferação e invasão de células da próstata e estimula as vias celulares (TWU et al., 2014). O frequente curso crônico da infecção em homens torna possível que os parasitas ascendam à próstata e estabeleçam um local de inflamação (CAINI et al., 2014).

Alguns pacientes apresentam dor abdominal inferior e há evidências de que a infecção por *T. vaginalis* pode ser transmitida verticalmente levando a casos de infecções vaginais e respiratórias em neonatos (PETRIN et al., 1998; CARTER e WHITHAUS, 2008).

2.5.1 Diagnóstico da tricomoníase

O diagnóstico não pode ser baseado apenas na manifestação clínica, visto que a infecção poderia ser confundida com outras ISTs, levando ao surgimento de resultados falso-

negativos (PETRIN et al., 1998). Subestima-se a prevalência de infecção por *T. vaginalis* por meio da utilização frequente de técnicas com baixa sensibilidade, como o exame direto a fresco e Pananicolau (SORVILLO et al., 2001).

Os métodos de diagnósticos existentes para identificação do parasito são: microscopia ou montagem úmida (DONNÉ, 1836), o método de cultura, que é o padrão-ouro para o diagnóstico da tricomoníase (GARBER et al., 1987); o desenvolvimento do dispositivo *InPouch*, feito para melhorar o método de cultura (BOUCHEMAL et al., 2017); os esfregaços cérvico-vaginais analisados pela técnica de Papanicolaou (AVILÉS et al., 2001); o teste Affirm VPIII (Becton Dickinson, Sparks, MD), um teste de amplificação para rRNA que permite a detecção de *T. vaginalis* dentro de 30 a 60 minutos (BOUCHEMAL et al., 2017); e o teste rápido de detecção de antígeno OSOM TV (Genzyme Diagnostics, Cambridge, Massachusetts, EUA), um ensaio de imunocromatografia (GAYDOS et al., 2017).

2.5.2 Tratamento da tricomoníase

As drogas nitroimidazólicas, representadas principalmente pelo metronidazol (MTZ) e tinidazol, são usadas como agentes tricomonicidas desde a década de 1960, sendo o MTZ o melhor tratamento de escolha (KISSINGER, 2015). Esta classe de drogas é a única aprovada pela *Food and Drug Administration* (FDA) para tratamento da tricomoníase. Esses medicamentos são baratos e amplamente disponíveis na saúde pública, principalmente MTZ (MEITES, 2013). O MTZ pode ser usado como aplicação tópica, por via oral e via sistêmica (FORNA e GULMEZOGLU, 2003).

O uso de 5-nitroimidazóis abrangem uma série de efeitos colaterais como anafilaxia, erupções cutâneas e pustulosas, prurido, rubor, urticária e febre (PEARLMAN et al., 1996). Além diso, o MTZ não pode ser administrados nos primeiros meses de gestação, pois vários estudos e meta-análises mostraram que este fármaco causa efeitos teratogênico e/ou mutagênico em recém-nascidos e bebês (BURTIN et al., 1995; PIPER, MITCHEL e RAY, 1993; SHEEHY et al., 2015).

A dependência de uma única classe terapêutica é um problema, pois estudos mostram prevalência de isolados resistentes ao MTZ em uma escala entre 2,5 a 9,6%. Isto resulta em aumento na dosagem do fármaco, provocando aumento de citotoxicidade em pacientes infectados, resultando em outros problemas de saúde associados a inflamações que

podem desencadear o surgimento de tumores, por exemplo (DAS, HUENGSBERG e SHAHMANESH, 2005; KIRKCALDY et al., 2012; SCHWEBKE e BARRIENTES, 2006).

O MTZ pode ser considerado um pró-fármaco, visto que é necessária a ativação metabólica (KULDA, 1999). Seu mecanismo de ação se dá através da penetração nas células por difusão e ativação nos hidrogenossomos de *T. vaginalis* (PETRIN et al., 1998). Esta ativação se dá pela redução dos grupos nitro por ferroxidinas, as quais são encontradas apenas em organismos anaeróbios, mostrando assim sua toxicidade seletiva (MENDZ e MÉGRAUD, 2002).

De acordo com as Diretrizes de Tratamento de DST 2015 da CDC (*Centers for Disease Control and Prevention* Atlanta, GA, USA), os regimes recomendados para o tratamento da tricomoníase correspondem a 2 g de MTZ ou tinidazol por via oral dose única. O MTZ em gel é considerado menos eficaz do que tratamento oral desde que preparações tópicas não podem atingir níveis terapêuticos na uretra ou glândulas perivaginais. Como tratamento alternativo, a dosagem oral de 500 mg de MTZ pode ser usada duas vezes ao dia durante 7 dias (WORKOWSKI e BOLAN, 2015).

Ressalta-se ainda que não existem sistemas de vigilância para a identificação de isolados resistentes, podendo assim subestimar o número de casos. Além disso, a tricomoníase atinge de forma desproporcional pessoas na base da pirâmide socioeconômica e desta maneira, recebe poucos investimentos para o desenvolvimento de novas alternativas. No entanto, com a falta de tratamentos adicionais para esta infecção milhares de pessoas permanecerão infectadas com *T. vaginalis,* conforme já alertado pelo CDC), a tricomoníase é considerada uma infecção parasitária negligenciada (SECOR et al., 2014).

2.6 PRODUTOS NATURAIS

A utilização de produtos naturais de plantas com fins medicinais é uma prática extremamente antiga na humanidade (RODRIGUES et al., 2011; CHING et al., 2006; HENDRICH, 2006). Civilizações antigas como a chinesa e a egípcia já utilizavam produtos naturais na medicina tradicional, e o conhecimento sobre estes tem se propagado até os dias atuais. Os produtos naturais continuam desempenhando um papel essencial na indústria farmacêutica moderna, apesar da imensa variedade de moléculas derivadas da química

combinatória (NGO et al., 2013). Aproximadamente 40% dos fármacos utilizados na sociedade contemporânea foram desenvolvidos a partir dos produtos naturais (NEWMAN e CRAGG, 2012).

O prêmio Nobel de Medicina e Fisiologia de 2015 foi concedido aos cientistas William C. Campbell, irlandês, e Satoshi Omura, japonês, por criarem novas terapias para combater doenças causadas por vermes nematódeos e para YouYou Tu, chinesa, por desenvolver uma nova terapia contra malária. A pesquisadora chinesa isolou a artemisina a partir da planta *Artemisia annua*. Isto mostra o destaque que os produtos naturais exercem na indústria farmacêutica, principalmente no combate às parasitoses (THOMÉ, 2015).

O Brasil é o país mais rico em palntas, uma vez que este apresenta mais de 20% do número total de espécies do planeta. Fatores climáticos atrelados à grande extensão territorial, contribuem para a formação de zonas biogeográficas distintas, também chamadas de biomas. Dentre estes, destacam-se: a Floresta Amazônica, considerada a maior floresta tropical úmida do mundo; o Pantanal, com a maior planície inundável; os campos dos Pampas; o Cerrado exibindo savanas e bosques; a floresta tropical pluvial da Mata Atlântica; e a Caatinga, abrangendo a região semiárida (MMA, 2012).

Até o presente momento, são reconhecidas 46528 espécies para a flora brasileira, sendo 4754 de Algas, 33111 de Angiospermas, 1568 de Briófitas, 30 de Gimnospermas e 1346 de Samambaias e Licófitas. No bioma Caatinga, são descritas 127 famílias, com 693 gêneros que abrangem 2213 espécies e 53 subespécies, apresentando 154 variedades (FLORA DO BRASIL, 2018).

Essa posição única entre os biomas brasileiros não foi suficiente para garantir à Caatinga o destaque que merece. Pelo contrário, a Caatinga tem sido sempre colocada em segundo plano quando se discutem políticas para o estudo e a conservação da biodiversidade do país. A variação da estrutura da vegetação é condicionada pela topografia, perturbação humana e, sobretudo, por uma combinação da precipitação média anual e atributos do solo. A pluviosidade média anual é de 1175 mm; a diferença entre a precipitação do mês mais seco e do mês mais chuvoso é de 240 mm. O solo é raso e rico em minerais, mas pobre em matéria orgânica devido às características da região, bem como pedregoso, com fragmentos de rochas na superfície. Por isso, dificilmente armazena as águas das chuvas (TABARELLI, 2005).

A região apresenta uma rica biodiversidade que desperta interesse em diversas áreas como agricultura, silvicultura, pastoreio e indústria, principalmente a farmacêutica. A vegetação da Caatinga é amplamente utilizada pela população para o tratamento de diversas enfermidades do trato respiratório, gastrointestinal, geniturinário, circulatório, doenças infecciosas, parasitárias e doenças venéreas (ALMEIDA et al., 2006; AGRA et al., 2007).

As plantas da Caatinga despertaram a atenção de vários grupos de pesquisa, os quais demonstraram as seguintes atividades de extratos, óleos essenciais e compostos isolados: antioxidante (MELO et al., 2010; DAVID et al., 2007), antifúngica (FONTENELLE et al., 2008; BIASI-GARBIN et al., 2016), analgésica, anti-inflamatória (MENDES et al., 2010; PAIVA et al., 2013), antibiofilme e antibacteriana (TRENTIN et al., 2011; TRENTIN et al., 2013), inseticida (MELO et al., 2015; SILVA et al., 2015), leishmanicida (VILA-NOVA et al., 2012), tripanocida (SILVA-JÚNIOR et al., 2004) e tricomonicida (VIEIRA et al., 2017) . O grande interesse nestas plantas está na capacidade de se adaptar a condições edafoclimáticas extremas, que podem levar à produção de metabólitos secundários de elevada complexidade e com potencial farmacológico, distintos de plantas encontradas em diferentes regiões.

No Brasil, as plantas medicinais ganharam destaque pelo Ministério da Saúde através da Política de práticas complementares, sendo estas inclusive, utilizadas para a saúde da mulher. Muitas plantas e derivados utilizados em preparações popularmente conhecidas como garrafadas são empregadas em uma série de doenças, incluindo as sexualmente transmissíveis (SOUSA et al, 2012).

2.7 *Bredemeyera floribunda* Willd.

Bredemeyera floribunda Willd. é uma trepadeira lenhosa pertencente à Família Polygalaceae (Figura 2). Apresenta folhas simples e glabras medindo de 7-10 centímetros de comprimento. Suas flores são alvacentas, com colorações amareladas ou avermelhadas, perfumadas e reunidas em vistosas inflorescências. As raízes têm casca espessa quase esponjosa, amarga e espumígena quando agitadas com água. Popularmente é conhecida por pacari, manacá, botica-inteira, marfim-de-rama, paurendoso, pau-caixão, pau-gemada, laça-vaqueiro, raiz-de-cobra e raiz-de-joão-da-costa. Duas espécies deste gênero são encontradas no Ceará, *B. floribunda* e *Bredemeyera brevifolia* Klotzsch (MATOS, 2007).

Figura 2 – *Bredemeyera floribunda* Wild. – Polygalaceae

Fonte: https://www.kew.org (2018).

Infusões feitas a partir da raiz seca de *B. floribunda* são utilizadas na medicina popular no tratamento de infecções de pele, disenteria amebiana, reumatismo e hipertensão. Outro uso relevante na medicina popular atribuído a *B. floribunda* é o tratamento de picadas de serpentes (SILVEIRA, et al., 1995; PEREIRA et al., 1996). O extrato bruto da raiz de *B. floribunda* é usado popularmente como diurético. O estudo in vivo mostrou o efeito deste produto sobre o transporte tubular renal (BEVENINO et al., 1994).

O extrato bruto da raiz de *B. floribunda* possui uma mistura de saponinas. Estas fazem parte de um grupo de produtos naturais compostos por glicosídeos de esteróides ou de terpenos policíclicos. São moléculas anfifílicas que têm uma parte de sua estrutura com característica lipofílica (triterpeno ou esteróide) denominada aglicona ou sapogenina e outra parte hidrofílica (com um ou mais açúcares), as quais demonstram ações tensoativas, detergentes e emulsificantes. São substâncias de alta massa molecular (600 a 2000 Da) que apresentam estruturas com número variado de açúcares com cadeia linear ou ramificada e diversas agliconas (SCHENKEL et al., 2001).

As saponinas encontradas na família Polygalaceae são as triterpênicas, possuindo 30 átomos de carbono e núcleo triterpênico. Estes compostos geralmente são bastante solúveis em água e pouco solúveis em soluções apolares. Em sua extração utilizam-se álcoois, etanol, metanol ou misturas hidroalcoólicas, empregando-se técnicas de maceração, decocção, percolação ou extração exaustiva sob refluxo (SCHENKEL et al., 2001).

Estudos com extratos que contêm saponinas relatam ação sobre membranas celulares, modificando a permeabilidade ou provocando sua destruição, bem como atividade hemolítica, moluscicida, espermicida, anti-helmíntica, hipocolesterolemiante, anti-inflamatória, antiviral e antitumoral. Tradicionalmente, plantas contendo saponinas são usadas como expectorante e diurético (ZHANG et al., 2008; SCHENKEL et al., 2001).

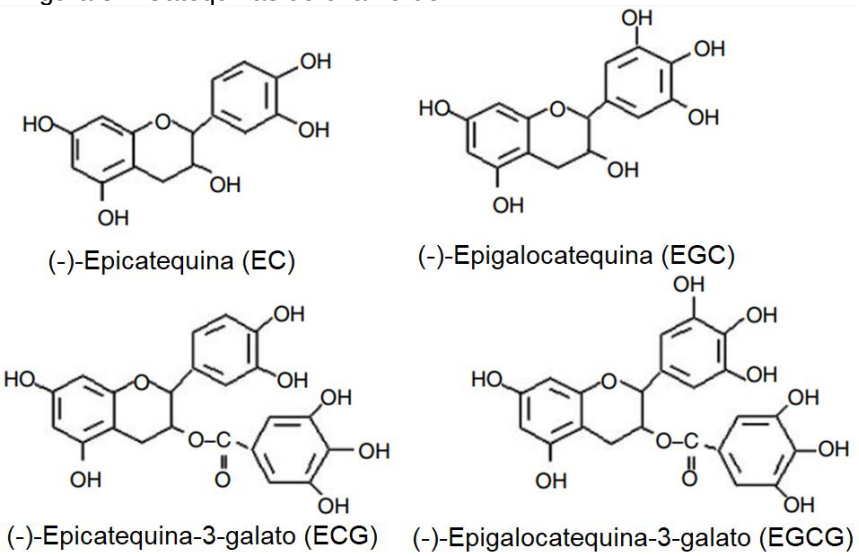
O extrato bruto de *B. floribunda* contém várias saponinas distintas, identificadas como saponinas triterpênicas e constituídas por ácido tenuilfólico e ácido bredemólico, sendo a estrutura deste último determinada por hidrólise ácida da saponina (BEVENINO et al., 1994). Uma saponina triterpênica nomeada bredemeyerosideo D foi isolada de *B. floribunda* e sua estrutura elucidada por Pereira et al. (1996), mostrando potente atividade antiofídica.

2.8 CHÁ VERDE

O chá é uma das bebidas mais populares consumidas em todo o planeta. É derivado das folhas de *Camellia sinensis* e produzido principalmente em quatro tipos (branco, verde, oolong e preto), dependendo das técnicas de oxidação e de fermentação aplicadas. Estudos epidemiológicos indicam que o consumo de chá verde está associado com a redução de muitas doenças crônicas como doenças cardiovasculares, diabetes e diferentes tipos de câncer (GAO et al., 1994; ISO et al., 2006; KURIYAMA et al., 2006; KURAHASHI et al., 2008).

Os principais flavonoides presentes no chá verde são chamados de catequinas, os quais correspondem de 30 a 40% dos componentes sólidos. As principais catequinas do chá verde são (–)-epicatequina (EC), (–)-epicatequina-3-galato (ECG), (–)-epigalocatequina (EGC), e (–)-epigalocatequina-3-galato (EGCG) (Figura 3). EGCG, o mais abundante, representa aproximadamente 59% do total de catequinas, enquanto EGC constitui 19%, ECG 13,6%, e EC 6,4%.

Figura 3 – Catequinas do chá verde.



Fonte: Bigelow e Cardelli (2006).

EGCG é um típico composto fenólico flavona-3-ol com oito grupos de hidroxilas livres, tornando-o um composto bioativo que possui funções biológicas versáteis. Apresenta também a maior habilidade de eliminação de radicais livres entre os compostos fenólicos comuns, além de vários compostos tanínicos (CAI et al., 2006). Assim como outros compostos fenólicos, EGCG também tem baixa biodisponibilidade. A absorção e o metabolismo do EGCG no intestino são fundamentais para as suas bioatividades e benefícios para a saúde (DU et al., 2012).

Como um componente dietético, o EGCG possui um grande número de benefícios para a saúde, mostrando atividades antioxidante (DU et al., 2012), anti-inflamatória, anticancerígena (CHEN et al., 2016; KOH et al., 2011; BETTUZZI et al., 2006; CHEN e ZHANG, 2007; LI et al., 2016; LIU et al., 2016; MA et al., 2013) e antimicrobiana (TAYLOR, HAMILTON-MILLER e STAPLETON, 2005; YUN et al., 2015; REYGAERT, 2014).

Diversos testes clínicos estão cadastrados no *US National Institute of Health* utilizando EGCG. Os testes abrangem os efeitos do EGCG na doença de Huntignton, síndrome muscular de Duchenne, melhoria da função endotelial, endometriose, quimioprevenção, doenças associadas a neuro-desenvolvimento, entre outros. Além disso, algumas pesquisas mostram os efeitos do EGCG contra parasitos do gênero *Leishmania* (INACIO et al., 2014; REIS et al.,

2013; INACIO et al., 2012), *Trypanosoma* (VIGUEIRA et al., 2012; GÜIDA et al., 2007) e *Trichomonas* (NORITAKE et al., 2017).

3 RESULTADOS

3.1 NATURAL PLANT DERIVED PRODUCTS: PROMISSING CHOICE AGAINST *Trichomonas vaginalis*?

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Abstract

Trichomonas vaginalis is the flagellate protist that causes trichomoniasis, a sexually transmited infection that leads to serious damage to human health, such as abortion, infertility, cervical and prostate cancer, and increase the risk of HIV and HPV infections. Every year, World Health Organization estimates that millions of people are infected worldwide. Metronidazole is the best drug of choice in the trichomoniasis treatment. However, the increase in metronidazole-resistant parasites and undesirable side effects of this drug make the search for alternative chemotherapeutic approaches a priority for the management of trichomoniasis. In this context, natural sources, such as plants, offer an enormous pool of bioactive compounds that can play a vital role in the development of effective therapeutic leads for parasitic diseases. In this review we will provide an overview of the current status of natural plant derived products with anti-*T. vaginalis* activity as promising alternative drugs for the treatment of trichomoniasis.

1. Introduction

Trichomoniasis is a sexually transmitted infection (STI) caused by the protist parasite *Trichomonas vaginalis*. The World Health Organization estimates that there are approximately 498.9 million cases of curable sexually transmitted infection (STI) annually, and 276.4 million of those cases are caused by *T. vaginalis*, making trichomoniasis the most common non-viral STI in the world. [1]. This infection entails a number of serious consequences for both women and men [2].

In women, symptoms include edema and vaginal erythema, vulvar pruritus and irritation [3, 4]. In addition, they may present dysuria, abdominal pain and small hemorrhagic patches on the ectocervix mucosa are observed in some cases [3, 5]. Among women of childbearing age, STIs (excluding HIV infection) are secondary only to complications in pregnancy and childbirth as a cause of morbidity and mortality [6].

In men, symptoms are associated with epididymitis, urethritis, dysuria, burning sensation after intercourse and itching [7]. Infection with this parasite also increases the risk of acquiring HIV [2] and HPV [8], cervical cancer [9] and aggressive prostate cancer [10, 11, 12, 13, 14]. In addition, *T. vaginalis* can cause infertility in men and women [3, 15, 16].

About 80% of the patients are asymptomatic, regardless of gender, and should be considered as potential carriers of the parasite [2], which was categorized as a ree-merging pathogen [17].

The current treatment for trichomoniasis is still quite limited, limited to nitroimidazole compounds, recommended by the Food and Drug Administration (FDA, USA), such as tinidazole and metronidazole (MTZ), the latter being the best drug of choice. However, resistance of *T. vaginalis* to MTZ (about 10% of clinical cases) and reactions, such as epigastric pain, nausea, vomiting, diarrhea, headache, seizures, vertigo, pruritus, flushing, urticaria, pustular eruptions, fever, are problems that have hampered the treatment of infection. In addition, it is not recommended that women use the drug in the first three months of gestation, as it is able to cross a transplacental barrier and cause problems to the fetus [18, 19, 20, 21].

As there is no approval through alternative treatments, the only option for patients with resistance infections is to use higher and sometimes toxic doses of MTZ, which contributes to an increase in the occurrence of side effects [22]. With restricted therapy, an increase in adverse effects and resistance to emerging drugs, it is essential to develop new drugs against trichomoniasis, preferably different from the class of 5-nitroimidazoles already established [18].

Plants have been used efficiently against parasites of neglected diseases, such as: filarial nematodes that are responsible for lymphatic filariasis (*Brugia sp*, *Wuchereria bancrofti*) and onchocerciasis (*Onchocerca volvulus*); trematode parasites of the genus *Schistosoma*; *Trypanosoma cruzi*, that causes causing Chagas' disease, sleeping sickness (*T. brucei*) and leishmaniasis (a group of diseases caused by parasites of the *Leishmania* genus) [23].

In this context, natural products and their derivatives appear as promising alternatives for the treatment of trichomoniasis, because they constitute a rich source of active molecules

and of great potential due to its great variety of compounds against *T. vaginalis*, already confirmed in several studies [24, 25, 26, 27, 28, 29].

Therefore, In this review we will shed light on the application of natural plant derived products on trichomoniasis.

2. Materials and Methods

An integrative review was carried out, which consists of a broad literature review, which gathers and synthesizes publications, contributing to the understanding of a particular problem, providing support for evidence based practice, through informed knowledge [30].

The steps were delimited in order to search for the necessary articles in: formulation of the problem (keywords and inclusion criteria); search procedures (inclusion of relevant literature on the topic of interest); data evaluation (extraction of relevant information from selected articles); data analysis and interpretation (data integration process); and presentation of the review.

The research was conducted in the US National Library of Medicine (PubMed), ScienceDirect®, Scopus® and Scientific Electronic Library Online (SciELO) databases. The descriptors "*Trichomonas vaginalis*", "natural compounds", "natural products" and "anti-*Trichomonas vaginalis*" were used and are identified in the Descriptors in Health Science (DECs) and Medical Subject Headings (MESH). The Boolean operator AND was used to refine the search for the articles and to make it more specific, so that there was a cross between "*Trichomonas vaginalis*" AND "natural compounds", "*Trichomonas vaginalis*" AND "natural products".

Only articles with complete texts available in English, published between 2000 and December 2017, were included. The excluded studies were those that were not related to natural products and to *Trichomonas vaginalis*, also excluding those that were repeated in the databases, as well as such as dissertations and theses.

After the search, about 154 papers were identified, which were submitted to the exhaustive reading of their titles and abstracts by the authors, independently, to ensure rigor in the selection of those that met the inclusion and exclusion criteria established. At the end, 27

studies were selected for reading in full, the other articles were excluded because they presented contents related to genetics, physiology, cellular and molecular aspects, which escaped the proposed theme.

3. Results and Discussion

In recent decades, natural products have been an important source of new chemical entities. These have been used as drugs in folk medicine, which has aroused the interest of the pharmaceutical industry, mainly due to the few side effects. Of the 15 antiparasitic medicines approved by the health authorities between 1981 and 2010, more than 50% come from natural or derived products [27]. Table 1 summarizes the activity of some natural products against *T. vaginalis*, showing the different concentrations tested.

Table 1. Natural products against *Trichomonas vaginalis*

Plants	Family	Type	Inhibitory concentration	Reference
Quillaja brasiliensis	Quillajaceae	Saponins	0.25mg/ml[b]	[26]
Passiflora alata	Passifloraceae	Saponins	0.25mg/ml[b]	[26]
Pistacia lentiscus	Anacardiaceae	Essential oil	10.0 mg/ml[d]	[32]
Carica papaya	Cariaceae	Extract	5.6 µg/ml[a]	[34]
Cocos nucifera	Arecaceae	Extract	5.8 µg/ml[a]	[34]
Artemisia absinthium	Asteraceae	Essential oil	87.2 µg/ml[c]	[35]
Elaeodendron trichotomum	Celastraceae	Extract	0.46 µg/ml[a]	[38]
Neurolaena lobata	Asteraceae	Extract	1000.0 µg/ml[c]	[39]
Arbutus unedo	Ericaceae	Extract	500 µg/ml[d]	[40]
Hypericum polyanthemum	Hypericaceae	Extract	325 µg/ml[d]	[41]

Scutellaria havanensis	Lamiaceae	Wogonin	56.0 µM[a]	[42]
Eucalyptus camaldulensis	Myrtaceae	Ether extract	6.25 mg/ml[c]	[43]
Verbena sp	Verbenaceae	Extract	4.0 mg/ml[b]	[44]
Campomanesia xanthocarpa	Myrtaceae	Extract	4.0 mg/ml[b]	[44]
Polygala decumbens	Polygalaceae	Extract	1.56 mg/ml[b]	[45]
Rheum ribes	Polygonaceae	Extract	1.0 mg/ml[d]	[46]
Nigella sativa	Ranunculaceae	essential oil	2.0 mg/ml[e]	[47]
Sapindus saponaria	Sapindaceae	Water-ethanol extract	0.156 mg/ml[b]	[48]
Sapindus saponaria	Sapindaceae	Butanolic extract	0.156 mg/ml[b]	[48]
Sapindus saponaria	Sapindaceae	Saponins	0.078 mg/ml[b]	[48]
Manilkara rufula	Sapotaceae	Extract	1.0 mg/ml[d]	[49]
Amomum tsao-ko	Zingiberaceae	Essential oil	22.49 µg/ml[e]	[51]
Zingiber officinale	Zingiberaceae	Extract	93.8 µg/ml[a]	[52]

a IC_{50} half inhibitory concentration

b MIC minimum inhibitory concentration

c GI 100% growth inhibitory concentration

d Single concentration tested

e Minimum lethal concentration

3.1. Amaranthaceae

Molecular interactions have been explored between *T. vaginalis* carbamate kinase (TvCK) and the compounds identified by GC–MS analysis of the methanolic extract of *Apamarga kshara*. The virtual docking results indicated that neophytadiene exhibited strong binding to the catalytic domain of TvCK. On the basis of free energy of binding and inhibition constant, neophytadiene was found to be a better TvCK inhibitor than other compounds.

Besides, structures of these compounds could be used for designing therapeutic lead molecule against cervical erosion caused by *T. vaginalis* [31].

3.2. Amaryllidaceae

Alkaloids called lycorine and candimine exhibited potential against *T. vaginalis*, reducing the viability of the parasite by about 60% in concentrations between 71.8 and 86.2 µg/ml [24].

3.3. Anacardiaceae

As regards *Pistacia lentiscus* (native to Mediterranean areas) mastic treated culture, it showed 100% inhibition of the parasitic growth at concentration of 15 mg/ml after 24 h incubation. It was observed 90% inhibition of the parasitic growth with concentration of 10 mg/ml after 24 h and complete inhibition of growth (100%) after 48 h. As well as 44% inhibition of the parasitic growth was observed with concentration of 5 mg/ml after 24 h, 85.6% after 48 h and 96.4% after 72 h till complete inhibition of growth (100%) after 96 h [32].

3.4. Apocynaceae, Cardiopteridaceae, Oleaceae and Solanaceae

Glycosides and aglycones isolated from *Solanum torvum*, *Plumeria obtuse*, *Gonocaryum subrostratum* and *Ligustrum confusum* showed anti-*T. vaginalis* activity in strains sensitive to metronidazole with MIC between 6.5 and 12.5 µM [33].

3.5. Arecaceae and Caricaceae

Crude methanolic extracts of 22 Mexican medicinal plants were tested against *T. vaginalis*. Among the plants tested, *Cocos nucifera* and *Carica papaya* presented the best trichomonicidal activity with IC_{50} values of 5.8 and 5.6 µg/ml, respectively [34].

3.6. Asteraceae

Active compounds from the essential oil of *Artemisia absinthium* were tested against *T. vaginalis*. The data showed that the compounds have trichomonicidal effect with GI of 99.1% at 500 µg/mL, 87.4% at 250 µg/mL and 53.7% at 100 µg/mL (GI_{50} 87.2 µg/mL) [35].

Another work have carried out an in silico screening of 952 antiprotozoal phytochemicals with specific protein drug targets of *T. vaginalis*. Only 42 compounds showed notable docking properties to *T. vaginalis* methionine gamma-lyase and to *T. vaginalis* purine nucleoside

phosphorylase. The most promising ligands were polyphenolic compounds, and several of these showed docking properties superior to either co-crystallized ligands or synthetic enzyme inhibitors [36].

The nanoemulsion of *Micana cordifolia* was used at concentrations of 100, 500 and 1000 ppm and it was observed that the highest concentration showed an excellent effect against *T. vaginalis*, equivalent to the values of metronidazole, during 12, 24 and 72 hours of in vitro treatment [38].

Another study of the same family showed that among 79 extracts of American plants, only the aqueous extract from the leaves of *Neurolaena lobata* R. Br. (L.) presented a 100% inhibition rate of growth of the parasites at the concentration of 1000 µg/ml [39].

3.7. Celastraceae

Extracts of *Elaeodendron trichotomum*, a plant used in Mexico against infective diseases, showed an effect against *T. vaginalis* with IC_{50} of 0.46 µg/ml, while metronidazole showed IC_{50} of 0.12 µg/ml [37].

3.8. Ericaceae

Extracts from leaves of *Arbutus unedo*, a plant widely used in Turkish folk medicine, were used in activity against *T. vaginalis*. Among the extracts tested, only ethyl acetate showed efficacy against the parasite, presenting a growth inhibition rate (GI) of 100% at the concentration of 0.5 mg/ml [40].

3.9. Hypericaceae

Extract and isolated compounds of *Hypericum polyanthemum* showed trichomonicidal activity. IC_{50} values were 0.066, 0.239, 0.320 and 0.061 mg/ml for benzopyrans HP1, HP2, HP3, and phloroglucinol derivative uliginosin B, respectively. Among these, HP1 was considered the most promising because it did not present a cytotoxic effect in mammalian cells [41].

3.10. Lamiaceae

Wogonin, an active compound of the chloroform extract of *Scutellaria havanensis* Jacq., a medicinal herb native to Cuba, showed trichomonicidal activity with IC_{50} of 56 μM, while metronidazole showed IC_{50} of 3.64 μM [42].

Human test was performed to investigate the therapeutic efficacy of the *Mentha crispa* in women with *T. vaginalis* infection. The study counted 72 women, of whom 60 were selected. Of these, 30 were medicated with secnidazole and 30 with *M. crispa*. After a clinical treatment phase, there was no statistically significant difference because *T. vaginalis* infection was not detected in 96.6% of the secnidazole group and 90% of the *M. crispa* group [28].

3.11. Myrtaceae and Verbenaceae

Five extracts and fractions of *Eucalyptus camaldulensis* were tested against *T. vaginalis*, showing good activity against the parasite, especially those dissolved in ethyl acetate, with GI from 100% at 12.5 mg/ml [43].

Ten plants used by the Brazilian indigenous group, Mbyá-Guarani, were evaluated against seven different isolates of the parasite. *Verbena sp*. and *Campomanesia xanthocarpa* showed the highest activity with MIC of 4.0 mg/ml, abolishing 100% of the parasites after 4 h of treatment [44].

3.12. Passifloraceae and Quillajaceae

Saponins of the genus *Quillaja*, *Passiflora* and *Ilex* presented trichomonicidal activity; however, saponins of *P. alata* and *Q. saponaria* with MIC of 0.25 mg/ml were more prominent [26].

3.13. Polygalaceae

A study showed that after screening 44 aqueous extracts from 23 plants from the Caatinga, Brazil, only *Polygala decumbens* was effective against the parasite, showing 100% of the viability reduction at the concentration of 1.56 mg/ml [45].

3.14. Polygonaceae

Anti-*T. vaginalis* activity of *Rheum ribes* L., plant distributed in Iran and a few neighboring countries, revealed that no growth was observed after 24 and 48 h of incubation at 1 mg/ml concentration of all extracts and fractions [46].

3.15. Ranunculaceae

Nigella sativa L., a plant native to the Mediterranean region, exhibited 100% inhibition of *T. vaginalis* trophozoites growth in 24 h with a minimum lethal concentration of 2 mg/ml and 50 µg/ml for essential oil and metronidazole, respectively [47].

3.16. Sapindaceae

Extracts and saponins of *Sapindus saponaria* apresentaram atividade tricomonicida com MIC of 0.156 mg/ml for extracts, and 0.078 mg/ml for saponins against a clinical strain; and 0.312, 0.156 and 0.078 mg/ml for extracts and saponins, respectively, against an ATCC strain [48].

3.17. Sapotaceae

Fractions from the *Manilkara rufula* extract, an endemic plant of the Brazilian Caatinga, inhibited more than 90% of the viability of *T. vaginalis* sensitive to metronidazole at a concentration of 1.0 mg/ml [49].

3.18. Theaceae

Black tea extract showed to be effective in inhibiting the growth of trichomonadines, showing better results against *T. vaginalis* (87.1% inhibited trophozoites). Strains resistant to metronidazole were also inhibited by black tea extract [50].

3.19. Zingiberaceae

Essential oil of *Amomum tsao-ko*, a plant commonly used in traditional Chinese medicine showed minimum lethal concentration and IC_{50} at 44.97- µg/mL and 22.49 µg/mL for clinical strain [51].

Extract of *Zingiber officinale*, ingredient widely used in traditional medicine showed activity against *T. vaginalis*. The IC_{50} after 24 hours of treatment was 93.8 µg/ml and 0.0326 µg/ml for ginger and metronidazole, respectively [52].

3.20. Solanaceae

A preliminary screen showed that tomatine at 100 µM concentration completely inhibited the growth of *T. vaginalis*. The IC_{50} value for *T. vaginalis* was 7.9 µM. Although the inhibition by tomatine was not as effective as that of the medicinal drug metronidazole, the relatively low IC_{50} values for *T. vaginalis* indicated tomatine as a possible natural alternative therapeutic for trichomoniasis in humans [53].

4. Perspectives

The most prevalent families that showed trichomonicidal activity were Anacardiaceae, Asteraceae, Ericaceae, Verbenaceae, Polygalaceae and Ranunculacea (each represented by a species) and Myrtaceae and Compositae (each represented by two species). Most of the activities tested against *T. vaginalis* used extracts.

Little is known about the mode of action of natural compounds on *T. vaginalis*, since much of the research is limited to percentages of parasite growth inhibition and cytotoxicity assays.

More information on the molecular mechanism involved in the action of natural compounds is necessary to predict side effects and analyze the effect of these in resistant strains. Just one research approached about molecular docking investigation involving natural products in this review.

5. Conclusions

Although trichomoniasis is not considered a neglected disease according to the World Health Organization, the increase in the number of cases has attracted the attention of researchers to the disease as a major public health problem. It is noticed that several tests have used natural products against *T. vaginalis*, However, the interest in the investment in this area is still small. Although the literature reports on several cases with excellent in vitro activities, it is observed that research with clinical trials is still extremely limited. Although metronidazole acts as the "gold standard" for treatment, several cases of toxicity are reported. Some natural products reported in the literature have 100% inhibition power at certain concentrations over a 24 h period and without toxicity to human cells. This shows that natural products would be excellent promising as a therapeutic alternative for trichomoniasis, especially in cases of drug resistance.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Funding Statement

This work was suppoted by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Instituto Aggeu Magalhães (IAM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We thank to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for funding research and Instituto Aggeu Magalhães for the place assigned to carry out the research (IAM).

Data Availability

All relevant data are within the paper and its Supporting Information files.

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3.2 EXTRACT OF THE *Bredemeyera floribunda* ROOTS INHIBITS THE PROLIFERATION AND CAUSES ULTRASTRUCTURAL CHANGES IN *Trichomonas vaginalis*

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ABSTRACT

Trichomonas vaginalis is an important human parasite that causes trichomoniasis, a widespread sexually transmitted infection. Currently, the treatment of choice for *T. vaginalis* infections is metronidazole (MTZ). MTZ is known to cause undesirable side effects, and MTZ-resistant parasites have been reported. Thus, the development of alternative treatment is recommended. Here, the antiproliferative and ultrastructural effects of extract of *Bredemeyera floribunda* roots against *T. vaginalis* were investigated. We observed a dose-dependent effect with an IC_{50} of 0,075 mg/mL after 24 h The most significant alterations were membrane shedding or disruption, bublle formation, wrinkled cells and the decrease of parasite clusters. In addition, autophagic vacuoles, abnormal Golgi swelling damaged hydrogenosomes and endoplasmic reticulum expansion were also observed. In addition, the quantitative analyses of the viability assays using combined markers of live and dead cells demonstrated that treatment with 0.075 and 0.15 mg/mL of *B. floribunda* did not significantly reduce the number of viable parasites compared with untreated cells. The extract showed no damage to the erythrocyte membrane at the concentration of 0.1 mg / mL, nor did it produce a reduction in the cellular viability of HeLa at the same concentration. Taken together, these results suggest that the extract of *B. floribunda* could be promising in the development of novel chemotherapeutic approaches against *T. vaginalis*.

Key words: Trichomoniasis; natural products; ultrastructure; cell death; membrane alterations.

1. INTRODUCTION

Trichomoniasis is the most common non-viral sexually transmitted infection in the world, caused by the protist *Trichomonas vaginalis*, an extracellular parasite that infects the human urogenital tract (WHO, 2012). Trichomoniasis is mainly observed in women, with symptoms ranging from an asymptomatic presentation to copious, malodorous discharge and punctate epithelial lesions known as strawberry cervix. Men are typically asymptomatic,

although some individuals present urethritis, prostatitis and/or discharge (Twu et al., 2014; Zhu et al., 2016; Sutcliffe et al., 2012). In recent years, this pathogen has been recognized to have significant implications for global public health. Serious adverse reproductive health outcomes, including pregnancy complications, pelvic inflammatory disease, preterm birth, low birth weight, abortion and infertility have been linked to *T. vaginalis* infection (Muzny e Schwebke, 2013). In addition, *T. vaginalis* has emerged as a cofactor for high-risk human papillomavirus (HPV) infection as well as the transmission and acquisition of the human immunodeficiency virus (HIV) and cervical and prostate cancer. These severe complications and high incidence of infection underscore the need to identify new drug targets and to advance vaccine development (Kissinger, 2015; Meites et al., 2015; Silver et al., 2014).

At present, *T. vaginalis* infections are treated with metronidazole [1-(2-hydroxyethyl)-2- methyl-5-nitroimidazole] (MTZ), and related derivatives of 5-nitroimidazole. These compounds selectively kill anaerobic microorganisms such as *Trichomonas* spp., *Giardia* spp. and *Entamoeba* spp (Meites, 2013; Kissinger, 2015). Although MTZ cure rates is approximately 95%, an increase in metronidazole-resistant trichomoniasis has been observed (Das, Huengsberg e Shahmanesh, 2005; Schwebke e Barrientes, 2006; Kirkcaldy et al., 2012). Moreover, several side effects such as allergic responses, hypersensitivity and nausea are associated with MTZ administration. Thus, the development of an alternative treatment is recommended. To date, the search for antiparasitic drugs has focused on the identification of active natural products, the identification of parasite targets and the use of available compounds active against other pathogenic microorganisms (Moraes et al., 2012).

In this context, *Bredemeyera floribunda* Wild. (Polygalaceae) is a perennial shrub native to the South America, commonly used in Brazilian folk medicine for the treatment of syphilis, cutaneous infections, amoebic dysenteries, and rheumatism (Matos, 2007). The infusion of the roots of the bush powder is suggested as a potent diuretic and is useful in the control of renal and hypertensive crises. The study of plants used by folk medicine is important to interconnect traditional medicine to the biotic environment preserving popular knowledge (Silveira et al., 1995; Pereira et al., 1996). Most of the saponins of Polygalaceae family were isolated from the extract of the roots of the plants obtained from ethanol/water 70%. The saponins in this family have characteristic structure composed of oxygenated pentacyclic triterpenoids (Lacaille-Dubois et al., 2005). Three saponins from *B. floribunda* have already been isolated, known as bredemeyerosides B, C and D (Pereira et al., 1996).

Considering popular ethnopharmacology as a basis for identification of plants with antiprotozoal activity and the need for new safe and effective drugs for the treatment of trichomoniasis, the aims of the present study is to investigate the *in vitro* antiproliferative effects and ultrastructural alterations of *B. floribunda* extract on *T. vaginalis*.

2. MATERIAL AND METHODS

2.1. Plant Material

The roots of *B. floribunda* Willd used in the study were collected in the Chapada do Araripe, Ceará, in March 2013. The plant was identified by comparison with a specimen of *B. floribunda*, collected in July 2001 in Macaíbas, Crato region, Ceará, and deposited in Herbarium Prisco Bezerra, Federal University of Ceará, with exsicata number 30844.

2.2. Obtaining extract

After the collection of the roots of *B. floribunda*, these were ground and placed in mariote containing methanol/water. Afterwards the plant residue was filtered with cotton, to remove larger particles, and the solvent evaporated under reduced pressure, giving rise to the hydroalcoholic extract called BFRMA.

2.3. Phytochemical characterization of *B. floribunda* extract

Thin layer chromatography was performed to detect the presence of secondary metabolite groups in the extract using specific chemical developers (color assays), namely: alkaloids - Dragendorff reagent and Mayer reagent; Flavonoids and cinnamic derivatives - Neu / PEG 400 reagent and aluminum chloride; Proanthocyanins and proanthocyanidins - hydrochloric vanillin; Triterpenes, carotenoids and steroids - Liebermann-Burchard reagent; Mono- and sesquiterpenes - phosphomolybdic acid; Anthrones, anthraquinones and coumarins - Bornträger reagent; Saponins - Liebermann-Burchard reagent (WAGNER and BLADT, 1996).

2.4. Parasite and cell culture

Three strains of *T. vaginalis*, two susceptible (JT and FMV1) and one MTZ-resistant (ATCC 50143) were used. Trophozoites were cultivated in TYM Diamond's medium supplemented with 10% heat inactivated fetal bovine serum (FBS). The cells were grown for

24 h at 37 °C, which corresponds to the logarithmic phase of the growth. HeLa cells (ATCC-CCL-2) were cultured at 37 °C in a 5 % CO_2/air mixture in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 10 % heat-inactivated FBS and 50 mg/mL gentamicin. The human cell lines passage was performed twice a week.

2.5. In vitro antiproliferative activities

Growth experiments with *T. vaginalis* JT trophozoites were initiated with 1×10^4 or 1×10^5 cells/mL. After 12 h of parasite growth, different concentrations of crude extract of *B. floribunda* (0.010 to 0.5 mg/mL) were added from stock solutions at 100 mg/mL in dimethyl-sulfoxide (DMSO). The final DMSO concentration never exceeded 1 % (v/v) and did not have any effect on parasite growth, viability or ultrastructure, as demonstrated by SEM, TEM and several viability assays. In all experiments, the cells were maintained in TYM medium containing 10 % FBS at 37 °C. Cell densities were determined using a hemocytometer and a light microscope. The activity of BFRMA was performed *in vitro*.

JT strain at 1.0×10^5 trophozoites/mL, was treated with different concentrations (0.1, 1.0, 2.5 mg/mL) and incubated for 24 h at 37 °C. Two controls were carried out: negative control (trophozoites in TYM medium) and vehicle for solvation (DMSO). The number of viable organisms was accessed by counting using Neubauer Chamber. Trophozoite viability was expressed as percentage in comparison with untreated parasites after incubation, considering motility, typical morphology.

The graphs were elaborated from the counts in triplicates using the program GraphPad Prism 5 (USA). Three independent experiments were performed in triplicate. Subsequently, the IC_{50} calculation was performed by linear regression in Microsoft Excel.

2.6 Scanning electron microscopy

JT strain (untreated and treated with 0.075 and 0.15 mg/mL) were washed with PBS and fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The cells were then post-fixed for 15 min in 1 % OsO_4, dehydrated in ethanol and critical point dried with liquid CO_2. The dried cells were coated with gold-palladium to a thickness of 15 nm and then observed with a Jeol JSM-5600LV scanning electron microscope (Tokyo, Japan).

2.7 Transmission electronic microscopy

JT strain (untreated and treated with 0.075 and 0.15 mg/mL) were washed with PBS and fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The cells were then post-fixed for 30 min in 1 % OsO_4, dehydrated in acetone and embedded in Epoxy Embedding Medium kit (Sigma-Aldrich). Ultra-thin sections were harvested on 300-mesh copper grids, stained with 5 % uranyl acetate and 1 % lead citrate, and observed with Tecnai™ G2 Spirit BioTWIN transmission electron microscope.

2.8. Cell viability assays in *T. vaginalis*

Viability test for trophozoites of *T. vaginalis* was done using *The LIVE/DEAD® Viability/Cytotoxicity Assay Kit* (ThermoFisher Scientific) according to the manufacturer's instructions.

2.9. Cytotoxicity assays in mammalian cells

Parasite-host cell interactions were initiated as described above. At the end of the incubation periods, MTT (0.5 mg/mL in DMEM) was added to the remaining host cells and incubated for an additional 1 h at 37 ◦C. Afterwards, the medium was discarded, and 1 mL of an acid isopropanol solution (4 M HCl: isopropanol PA; 1:99, v/v) was added to each well to solubilize the colored formazan product that was formed. Absorbance was read at 570 nm, and the background was subtracted at 650 nm on GloMax®-Multi Microplate Multimode Reader (Promega). The viability was calculated with the following equation: 1 – (E/C). All measurements of experimental (E) samples (A570 nm-650 nm) were indexed to those of control (C) samples (E/C), which showed no loss of viability, and then subtracted from 1.0. All data points were performed in triplicate. The results are the average of three independent experiments.

2.10. Hemolytic activity

The hemolytic activity was investigated by incubating 100 µL of serially diluted BFRMA in saline solution (NaCl 0.85% + $CaCl_2$) with 100 µL of 2% red blood cells suspension (human O^+) for 3 h at 37 °C in a 96-well microplate (U-bottom shape) under constant agitation. The microplate was centrifuged at 1500 g for 4 min. Cell lysis was then measured

spectrophotometrically (540 nm). Erythrocytes in saline or treated with Triton X-100 were used as negative and positive control of hemolysis, respectivelly. The results were determined by the percentage of hemolysis compared to the positive control (100% hemolysis) and negative control (0% hemolysis), and the experiments were performed in quadruplicate in two independent assay.

2.11. Statistical analysis

The results for all assays are the average of three independent experiments performed at least in duplicate. Statistical comparison was performed using ANOVA test and using computer analysis (GraphPad Prism v. 5.00, California, USA). $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Phytochemical characterization of *B. floribunda* extract

The substances present in the crude extract of *B. floribunda* are listed in the table below (Table1).

Table 1. Group of secondary metabolites present in BFRMA

Group of secondary metabolites	Presence/Absence
Flavonoids	+
Saponins	+
Sugars	+
Quinones	-
Coumarins	-
Triterpenes	-
Steroids	-
Mono and sesquiterpenes	-
Alkaloids	-

+ presence; - absence

3.2. Antiproliferative effects

Initially, growth curves were performed using two different parasite densities (10^4 and 10^5 cells/ml) to determine whether the initial cell density affects the action of the extracts on the parasite proliferation. In both conditions, the effect on growth was dose dependent. At 60 h of growth, it was observed that at some points on the curve with a concentration of 10^4 cells/mL, the cells were still in the log phase, while some points in the curve at the concentration of 10^5 cells/mL, the cells were already in phase stationary and declining (Figure 1), causing this latter concentration to be chosen for the later tests. The IC_{50} value for BFRMA-treated cells after 24 h was 0.075 mg/mL, as determined by linear regression ($y = 12.59x$, $R^2 = 0.9221$). Cells incubated at the concentration of IC_{50} and 2x IC_{50} were subsequently used for viability and ultrastructural analyzes.

The BFRMA extract was tested in two other strains (one clinical isolate and one MTZ resistant isolate). Results showed that BFRMA reduced the viability of FMV1 strain (Figure 2B) by more than 97%, JT (Figure 2A) and ATCC 50143 strains of *T. vaginalis* (Figure 2C) in 80% at 0.1 mg/mL.

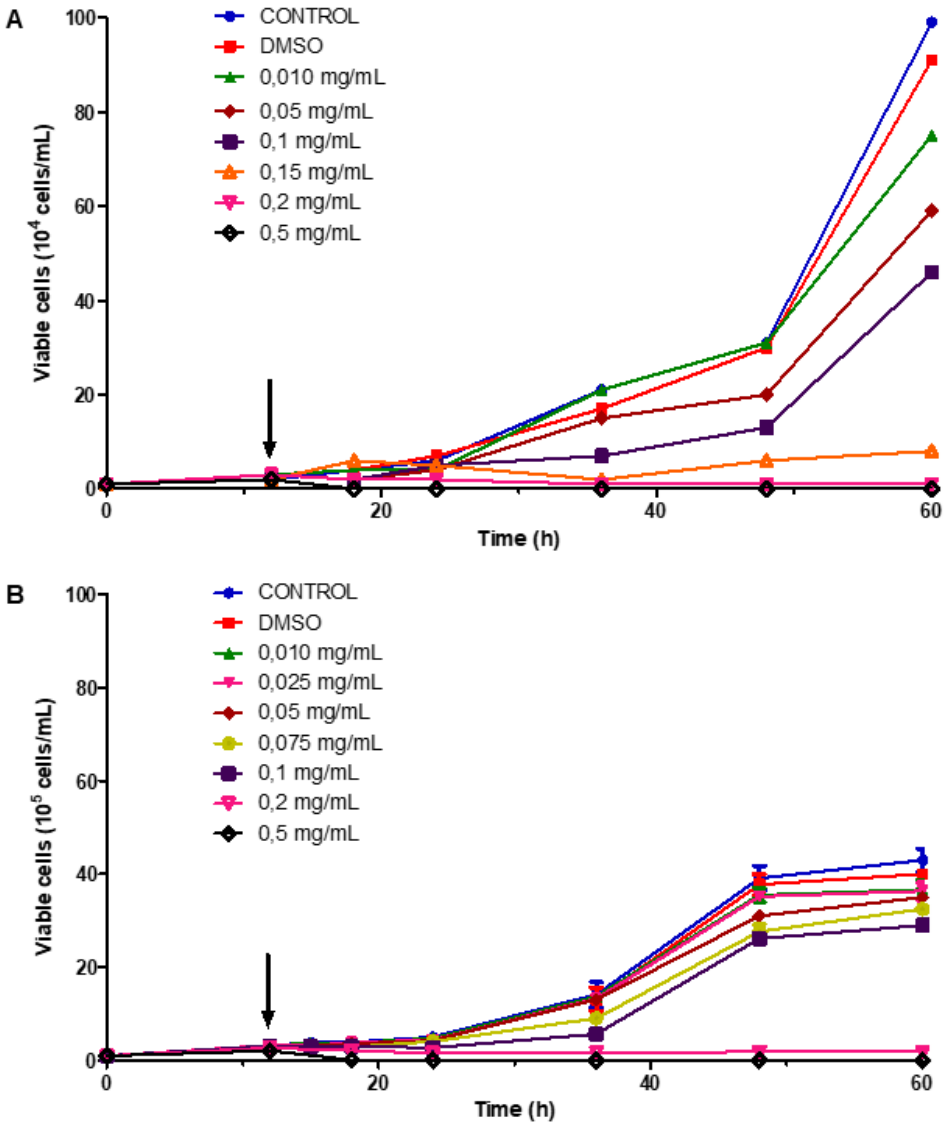

Figure 1. Growth curve of *T. vaginalis* under effect of different concentrations of *B. floribunda* extract at 10^4 cells/mL (A) and 10^5 cells/mL (B). The parasites were initially cultured for 12 h at 37 °C (initial inoculum: $1x10^4$ (A) and $1x10^5$ (B) parasites/mL). After this period (arrow), 0,010 to 0,5 mg/mL of *B. floribunda* were added to the culture medium and the parasites were incubated for up to 48 h at 37 °C. The cell growth was calculated after 6 to 48 h of incubation. Parasites incubated with 0.2 % DMSO were used as a control. Values are expressed as the means ± SD in three independent experiments, each performed in triplicate.

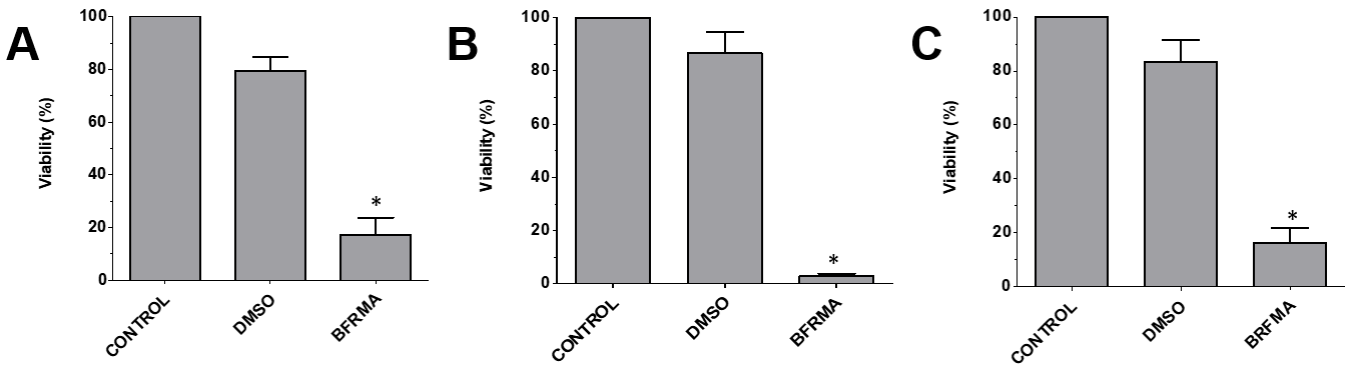

Figure 2. Activity against *Trichomonas vaginalis* (A – JT strain; B – FMV1 strain; C- ATCC 50143 strain) of the crude extract from *Bredemeyera floribunda* at 0.1 mg/mL. Bars represent the mean ± SD of three different experiments (parasite suspensions) performed in triplicate. *p<0.001 compared to control.

3.3. Ultrastructural alterations

To determine the effects of BFRMA on the morphology and fine structure of *T. vaginalis*, parasites treated with 0.075 or 0.15 mg/mL of extract for 24 h were observed using electron scanning microscopy (SEM) and transmission electron microscopy (TEM). Untreated *T. vaginalis*, grown in axenic medium, is characterized by a pear-shaped body, four anterior flagella, an undulating membrane reaching the posterior end of the cell body and a recurrent flagellum continuing beyond the undulating membrane by a free-trailing portion that is apparent using SEM (Figure 3A). By TEM, one anterior nucleus, the pelta-axostyle complex, a well-developed Golgi complex, hydrogenosomes, and an endoplasmic reticulum sparsely found around the nucleus and in close association with the hydrogenosomes are observed in the parasite (Figure 3B).

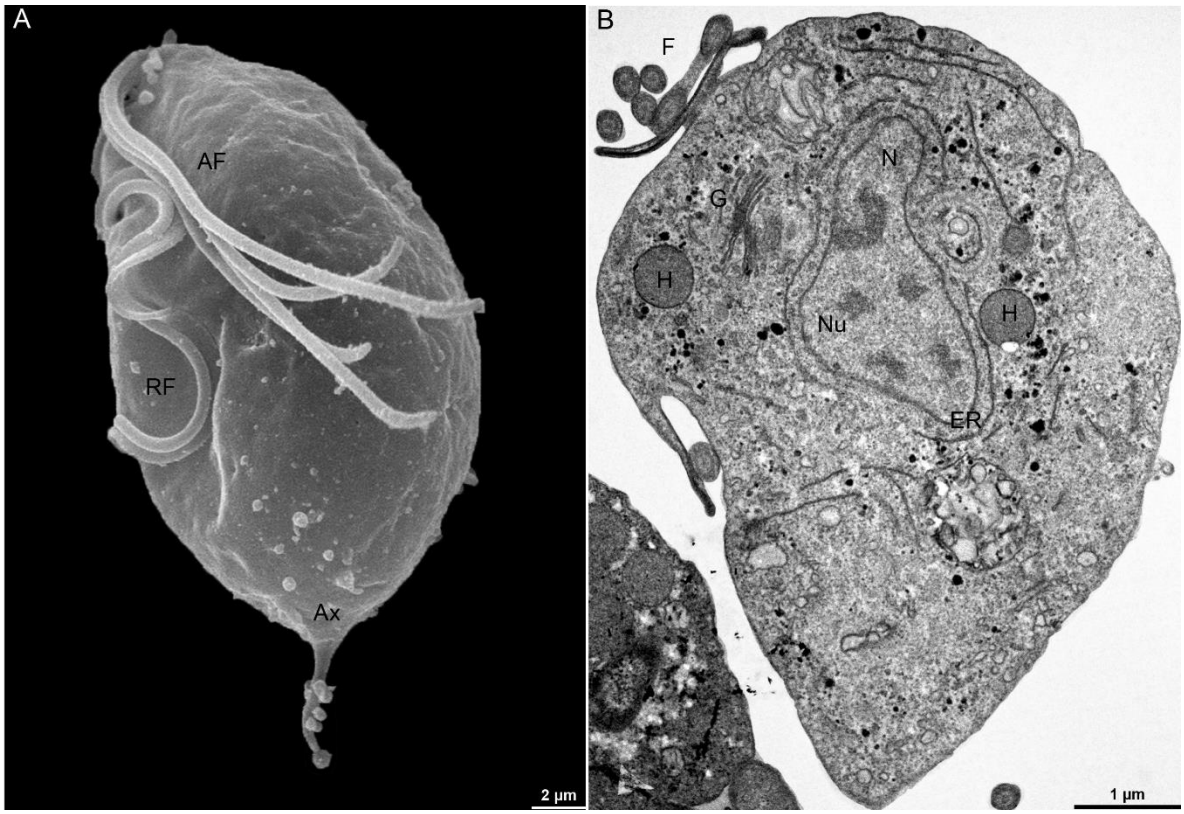

Figure 3. Fine structure of untreated*T*. *vaginalis*. (A) SEM. Parasite exhibits four anterior flagella (AF) and one recurrent flagellum (RF). The axostyle (Ax) tip is visible. (B) TEM of a longitudinal section of the parasite. The parasite displays one anterior nucleus (N), Nucleolus (Nu), Golgi complex (G), hydrogenosomes (H), and endoplasmic reticulum (ER). Bars at A 2 µm and B 1 µm.

After treatment with BFRMA at the IC_{50}, changes in cell morphology were observed, with the appearance of membrane shedding and projections (Figure 4A and 4B), enlargement (giant trophozoites), rounded and wrinkled forms, and pseudocyst formation (internalisation of flagella under stress conditions). SEM analysis showed that approximately 50% maintained pear-shaped morphology, 10% exhibited shedding/membrane projections, 13% were wrinkled and 27% showed other changes (cell lysis, rounded forms, pseudocysts and shoots) for both concentrations (IC_{50} and 2x IC_{50}) (Fig. 5).

The BFRMA treatment in *T. vaginalis* provoked the appearance of several morphological alterations indicative of cell death, including (a) plasma membrane blebbing (Figure 4A, B and E), (b) wrinkled cells (Figure 4A, C, D, E and F), (c) concentric membrane whorls, which resembled autophagous (Figure 5A and D), (d) abnormal Golgi complex reduction (Figure 5B), (e) hydrogenosomes eletronlucent (Figure 5E), (f) intense cytosolic vacuolisation (Figure. 5A and F), (g) plasma membrane disruption and lysed cells (Figure 4C),

(h) budding (Figure 4D) and (i) rounded cells (Figure 4F). Ultrastructural changes in the nucleus, such as nucleoid and vacuolisation, were also observed in both concentrations (Figure 5C).

In SEM it was observed that the untreated (control) cells were forming clusters, and absence of changes in their typical morphology (Figure 6A). It was also noticed that clusters decreased, causing the trophozoites not to adhere to each other (Figure 6B). At the concentration of 2x IC_{50}, in addition to all the mentioned characteristics, trophozoite shoots appeared and a greater cellular disaggregation was observed (Figure 6C). The quantitative analysis showing the changes are shown in figure 7.

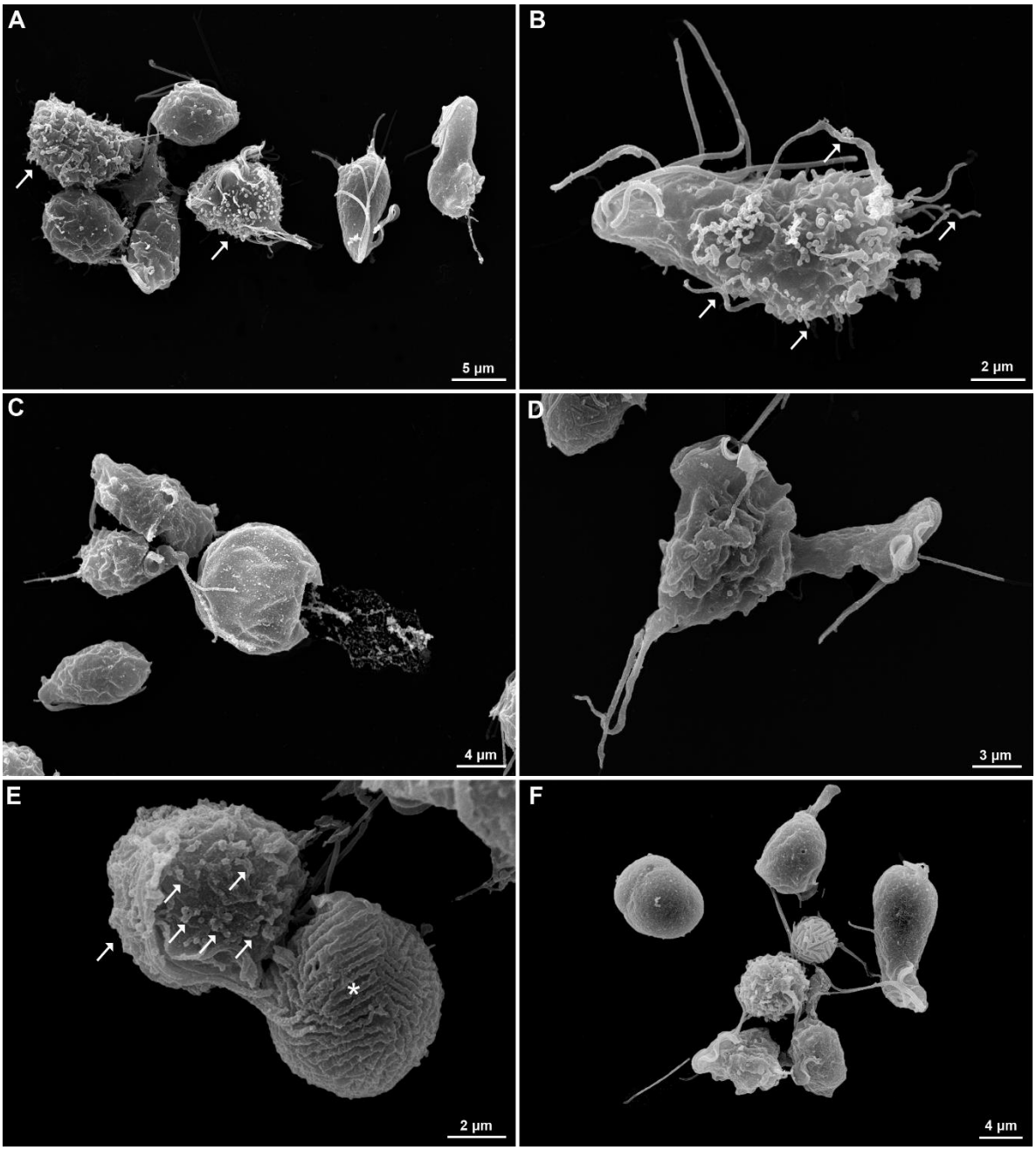

Figure 4. SEM of BFRMA-treated *T. vaginalis*. Parasites were incubated with 0.075 mg/mL (A – D) and 0.15 mg/mL (E, F) BFRMA for 24 h. Some parasites exhibit morphological alterations indicative of cell death, such as appearance of wrinkled cells (*) and membrane blebbing (arrows), as well as membrane disruption and cell lysis (C). Bars at 2 µm (B,E), 3 µm (D), 4 µm (C, F) and 5 µm (A) .

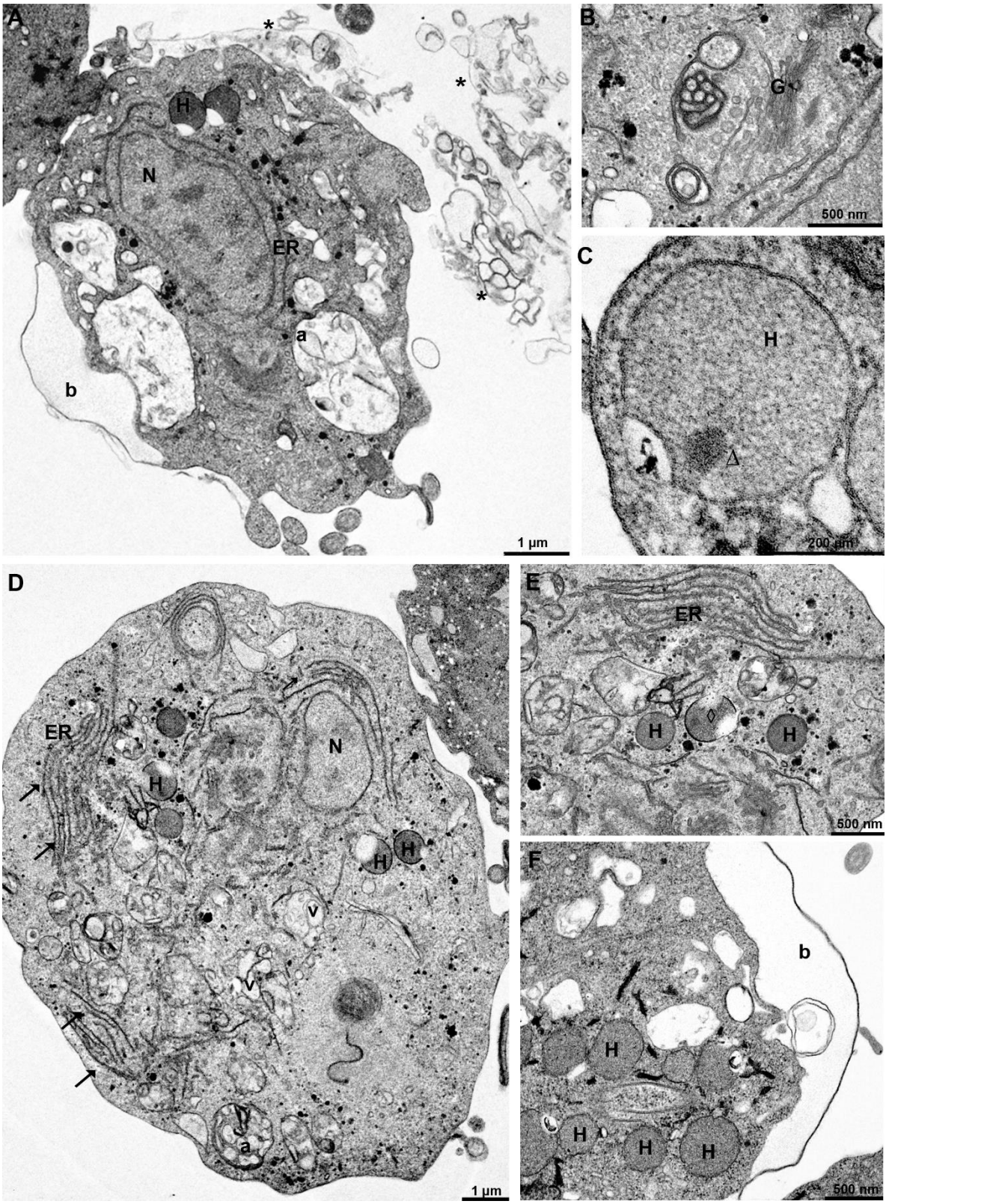

Figure 5. TEM of BFRMA-treated *T. vaginalis*. A, B and C – Parasites were incubated with 0.075 mg/mL and D, E and F - parasites were incubated with 0.15 mg/mL for 24 h. The parasites exhibit alterations indicative of cell death, such as endoplasmic reticulum expansion (arrows), autophagous (a), abnormal Golgi complex (G) swelling, hydrogenosomes eletronlucent (lozenge) with nucleoid (triangle), bubble (b) and cell debris (asterisks). Bars at A, D 1 µm, C 200 nm and B, E, F 500 nm.

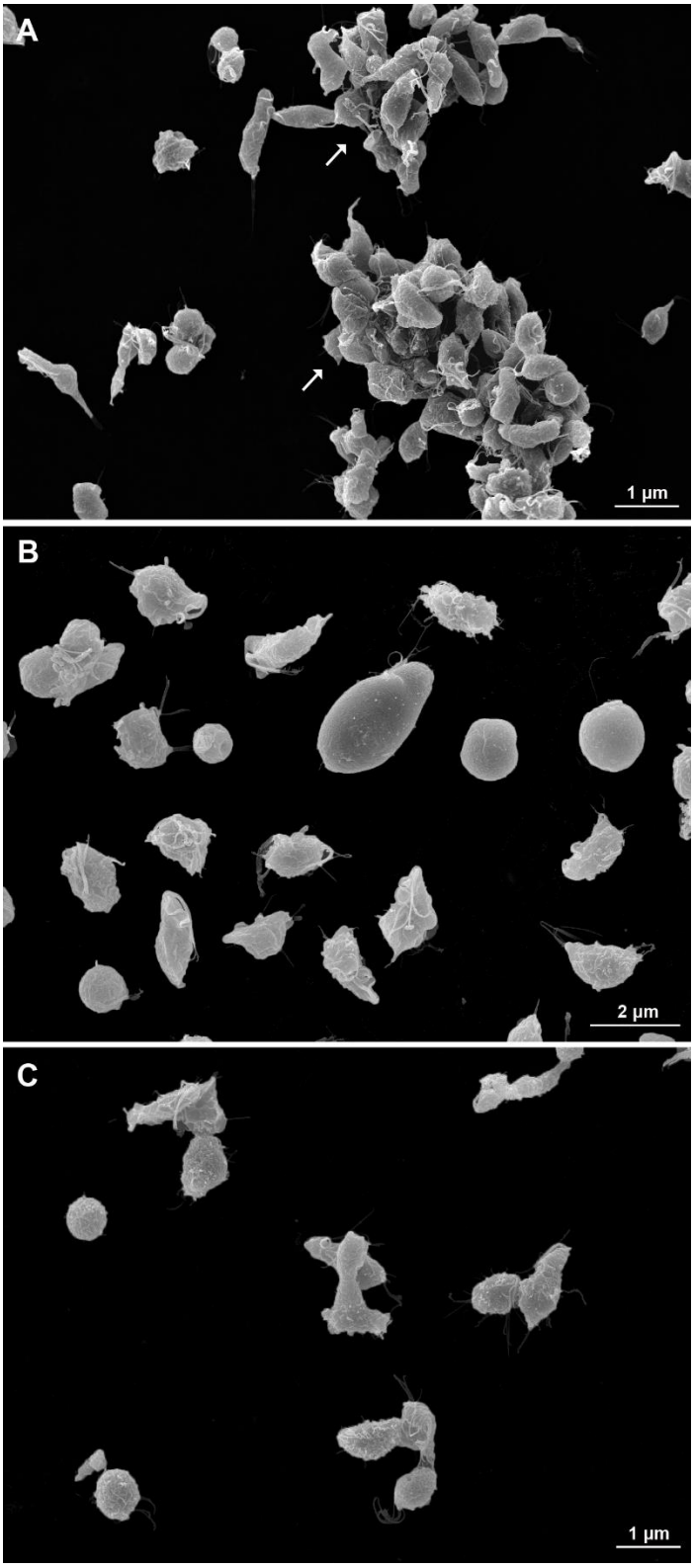

Figure 6. Overview of SEM of BFRMA-treated *T. vaginalis*. Parasites were incubated with 0.075 mg/mL (B) and 0.15 mg/mL (C) BFRMA for 24 h. The majority of the parasites are presenting typical morphology with presence of clusters (arrows). Some parasites exhibit morphological alterations as well as decrease of clusters in relation to the control. Bars at 1 µm (A, C) and 2 µm (B).

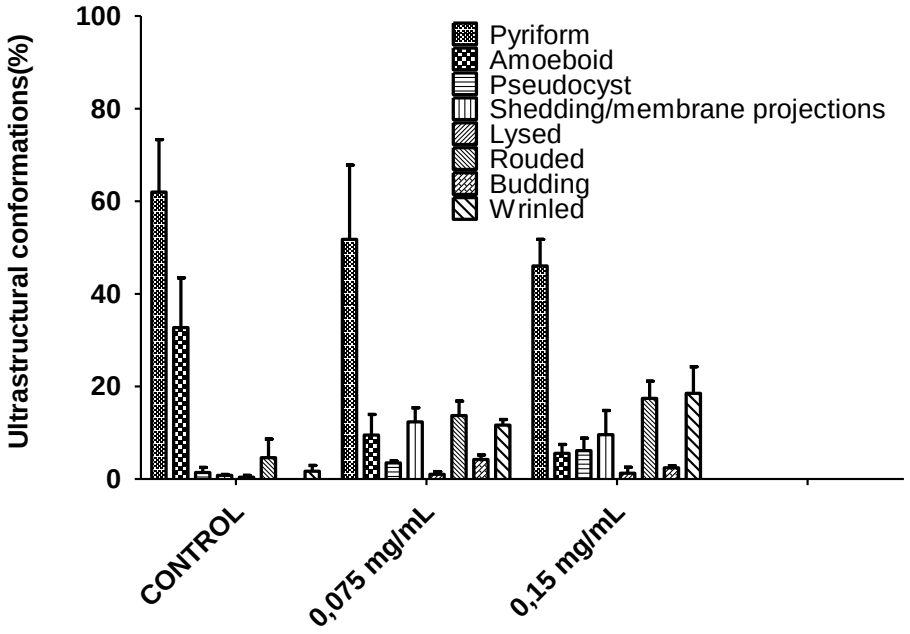

Figure 7. Quantitative analysis of cellular modifications from SEM treated with BFRMA at IC_{50} concentrations and double IC_{50}, in addition to the control with DMSO, at the highest concentration tested. Three independent experiments were performed in triplicate, with a minimum count of 500 cells. Data are expressed as means ± SD.

3.4. Cell viability test in *T. vaginalis*

After 24 h of culture growth, approximately 91,5 % of untreated parasites were viable (Fig. 10), as determined using LIVE/DEAD®, whereas about 86% of BFRMA-treated cells at IC_{50} and 88% of BFRMA-treated at 2x IC_{50} cells were viable. This indicates that the extract has an growth-inhibitory effect on the cells, but not provoke cell death (Figure 8).

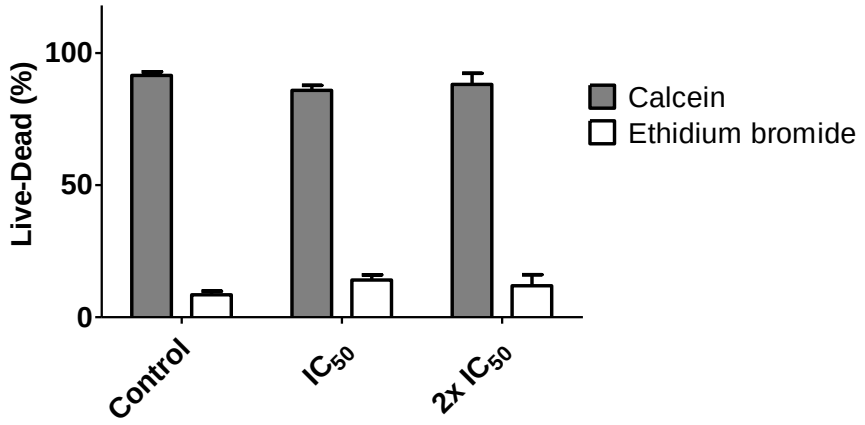

Figure 8. Percentage of the cellular viability of *T. vaginalis* after 24 hours of treatment with BRFRMA at IC50 and 2x IC50. Bars represent the mean ± SD of three different experiments.

3.5. Cytotoxicity assays in HeLa

Cytotoxicity assays were performed to evaluate the effects of the BFRMA extract on human cell lines. MTT assays showed that at the concentration of 0.1 mg/mL, HeLa cells did not have the viability affected. At concentrations of 1 mg/mL and 2.5 mg/mL, cytotoxicity of 54.25% and 100% was detected, respectively. At the concentration of 2.5 mg/mL, DMSO was also cytotoxic (Figure 9).

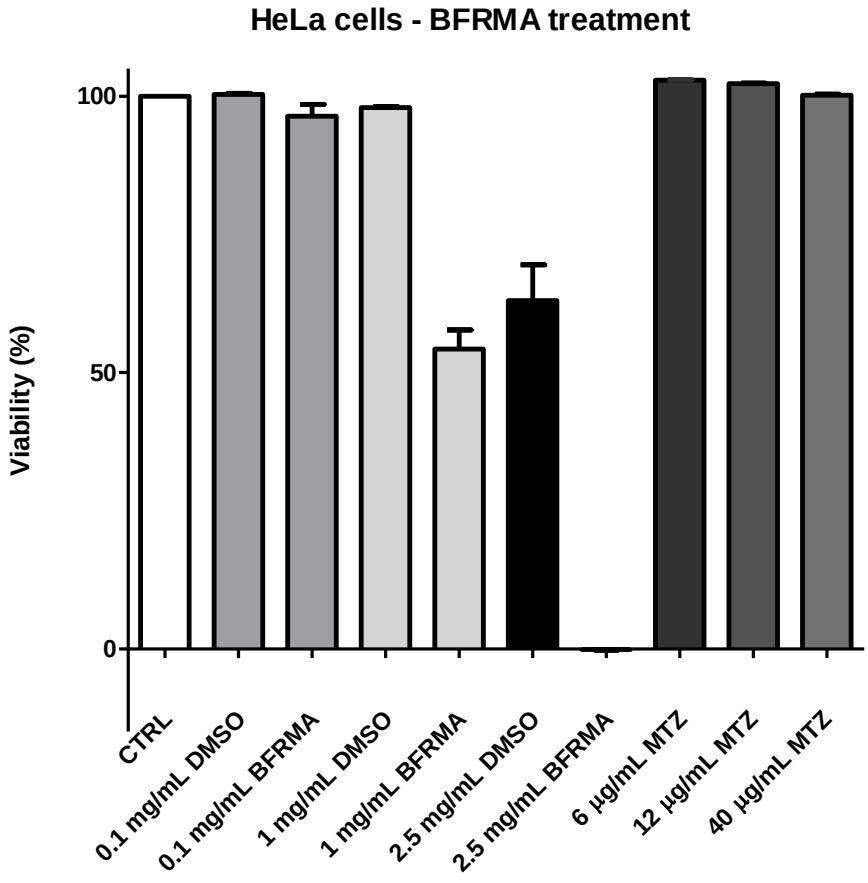

Figure 9. MTT viability assay of BFRMA. Results are expressed as the mean ± SD of three independent experiments that were each performed in triplicate. Note that only high concentrations of DMSO (i.e., 2,5%) significantly affected the viability of HeLa as compared to the control.

3.6. Hemolytic activity of *B. floribunda*

The hemolytic activity of *B. floribunda* was evaluated, and after 3 h of incubation, hemolysis was not observed at 0.1 mg/mL. In turn, BFRMA-treated erythrocytes presented approximately 90% of lyses after 3 h at 1.0 and 2.5 mg/mL (Figure 10).

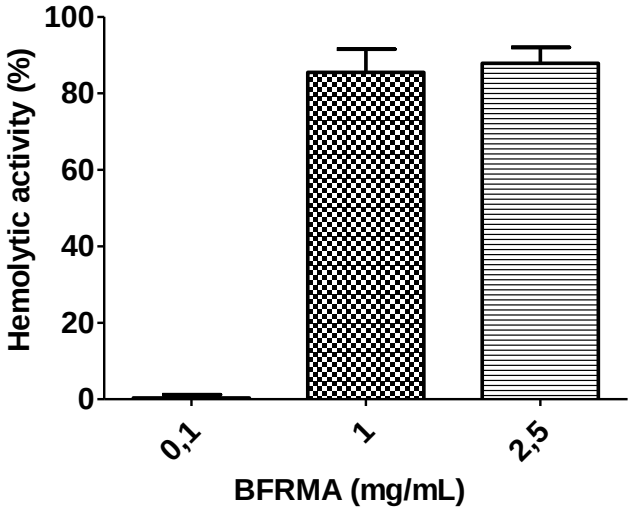

Figure 10. Hemolytic activity of *B. floribunda*. The results were expressed as the percentage of erythrocytes lysis. Values are expressed as the mean ± SD of three independent experiments that were each performed in triplicate.

4. Discussion

Extracts from medicinal plants have been widely used against several microorganisms as promising therapeutic alternatives for some diseases. In this context, several studies report the efficacy of plant extracts to some parasites, including *T. vaginalis* (Calzada et al., 2007, Lara-Diaz et al., 2009, Vital and Rivera, 2011; Frasson et al., 2012; Naidoo et al., 2013).

Many extracts display in their chemical composition components constituted by a hydrophobic triterpenic or steroid portion attached to one or more sugar chains, called saponins. Studies have shown that this chemical class has exhibited activities against *T. vaginalis* (Tiwari et al., 2008, Damke et al., 2013; Rocha et al., 2012).

In the present study, we evaluated ultrastructural changes as observed by different microscopy techniques in *T. vaginalis* after treatment with extract from roots of *B. floribunda*, that contains saponins and flavonoids in their chemical composition. Saponins exhibit a wide variety of biological effects acting as anti-inflammatory agents (Eskander et al., 2014),

anticancer agents (Man et al., 2010), antibacterial agents (Saleem et al., 2010), antiviral agents (Cinatl et al., 2003), anti-fungal and insecticidal agents (Brum Vieira et al., 2017).

In this context, saponins has shown in vitro inhibitory effects in *T. vaginalis*. The monodossomic saponins of *Hedera colchica* (Mshvildadze et al., 2000) and *Buddleja madagascariensis* (Emam et al., 1996) exhibited high anti-*T. vaginalis* activity, as well as the fractions enriched with monodesodic saponins of *Passiflora alata* and *Quillaja saponaria*, which were more active in that the bidesmosal saponins fraction of *Ilex brasiliensis* (Rocha et al., 2012) by inhibiting the parasite growth. Similarly, our data showed that the growth of *T. vaginalis* were inhibited after treatment with BFRMA and these results could be attributed, at least in part, by the action of the saponins.

T. vaginalis treated with BFRMA showed changes in hydrogenossomes, structures involved in molecular hydrogen production and also are related to ATP synthesis, essential for the energy metabolism of trichomonadine. Hydrogensomes are endosymbiotic organelles related to mitochondria. (Embley, 2006; Hjort et al., 2010; Rada et al., 2011; Schneider et al., 2011).

Studies with others parasites have demonstrated ultrastructural changes in the mitochondrial morphology. Promastigote forms of *L. amazonensis* treated with natural leishmanicidal agents, such as dihydroxy-methoxychalcone from *Piper aduncum* L. (Piperaceae) inflorescences showed these changes (Torres-Santos et al., 2009). In this study, the treatment with BFRMA induced ultrastructural changes in the trophozoites of *T. vaginalis*, which suggests that hydrogenosomes are involved in the action of these extract.

Our results show that BFRMA treatment induced anti- *T. vaginalis* effects with features typical of apoptosis (membrane shedding, nucleoid, intense vacuolization with cytoplasmic disorganization), autophagy and necrosis (plasma membrane disruption and lysed cells). Studies with others parasites showed atypical intense vacuolization with cytoplasmic disorganization in the parasites that were treated with others extracts showing the appearance of the autophagic vacuoles that are characteristic of cell death by autophagy (Brenzan et al., 2012; Rodrigues and Souza, 2008; Monte-Neto et al., 2011).

The evaluation of cytotoxicity through hemolytic activity tests proved to be an alternative screening method for simple toxicity. Assessment of toxicity is paramount when considering safe treatment. Hemolysis is characterized by rupture of erythrocytes with the

release of hemoglobin. The in vitro hemolysis test is used as a method to screen for substance toxicity (Aparício et al., 2005).

Saponins are secondary metabolites with a broad spectrum of biological effects and, depending on the chemical structures, mainly monodesmosic and bidesmosic, may have different effects (Gauthier et al., 2009). Erythrocytes lysis is a rather obvious activity of saponins, which may occur due to the amphiphilic nature of the compounds that interact with lipids, leading to pore formation and membrane rupture (Rocha et al., 2012; Augustin et al., 2011; Lacaille-Dubois and Wagner, 1996). Our study showed that as the concentration of BFRMA increased, hemolysis was more evident, showing the hemolytic effect can be caused by the saponins.

Considering the potential against *T. vaginalis* demonstrated by BFRMA, cytotoxicity to mammalian cells was evaluated. The extract exhibited a certain level of cytotoxicity against HeLa strains. However, in vitro cytotoxicity should not be the only criterion for deciding whether a compound should be set aside or referred to an animal model to continue the search for a new bioactive molecule. Studies indicate that flavonoids, vicenin and vitexin showed high in vitro cytotoxicity against different cancer cell lines; however, when vicenin fractions were tested in vivo, a protective effect was observed in the colon carcinogenesis process, suppressing both the initiation and the promotion of carcinogenesis (Mohammed et al., 2014; Fernandes et al., 2011).

5. Conclusions

Our results demonstrated that *B. floribunda* exhibits anti-proliferative effects on *T. vaginalis*. BFRMA extract induced several ultrastructural changes in the trophozoites of *T. vaginalis*. Ultrastructural changes such as cytoplasmic vacuolization, reticulum endoplasmatic expansion, membrane shedding and a rounded form of the parasites were observed. To use this extract as a drug, further studies should be performed involving other human cell lines, as well as the fractionation and isolation of new compounds from the extract.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Funding Statement

This work was suppoted by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Instituto Aggeu Magalhães (IAM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We thank to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for funding research and Instituto Aggeu Magalhães for the place assigned to carry out the research (IAM).

Data Availability

All relevant data are within the paper and its Supporting Information files.

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3.3 EFFECTS OF (-)-EPIGALLOCATECHIN-3-GALLATE (EGCG) ON THE *Trichomonas vaginalis* ULTRASTRUCTURE

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ABSTRACT

Trichomonas vaginalis is an important human parasite that causes trichomoniasis, a cosmopolite sexually transmitted disease. At present, the treatment of choice for *T. vaginalis* infections is metronidazole. However the increase in metronidazole-resistant parasites and side effects of this drug urgently require new chemotherapeutic alternatives for the treatment of trichomoniasis. In this study, the antiproliferative and ultrastructural effects of (-)-epigallocatechin-3-gallate (EGCG) against *T. vaginalis* were investigated. This catechin from green tea showed that extract induced changes in the ultrastructure of *T. vaginalis*. Alterations such as membrane shedding and disruption, wrinkled cells and internalization of flagella were the most significant. Further, autophagic vacuoles and damaged double membrane hydrogenosomes were also observed. EGCG showed activity against HeLa cells and anti-hemolytic effects. Therefore, these results suggest EGCG has potencial against *T. vaginalis* and contribute to the understanding of the pharmacological properties, as well as the rational development of alternatives for the treatment of trichomoniasis.

Key words: *Trichomonas vaginalis*; EGCG; ultrastructure; cell death; membrane alterations.

1. INTRODUCTION

Trichomoniasis is the most common non-viral sexually transmitted infection in the world, being caused by the protist parasite *Trichomonas vaginalis*. Several adverse effects on human reproduction have been reported from infection by this parasite, including pelvic inflammation, complications in pregnancy, preterm births, abortions, low birth weight and infertility (WHO, 2012; Hirt and Sherrard, 2015; Soper, 2004). Prolonged infections caused by the parasite have resulted in the beginning of prostate and cervical cancers (Sutcliffe et al., 2006), as well as the parasite being considered as a cofactor for human papillomavirus (HPV) infection (Noel et al., 2010) and acquired immunodeficiency virus (HIV) (Van der Pol et al., 2008).

Since the 1960s, metronidazole [1- (2-hydroxyethyl) -2-methyl-5-nitroimidazole] and 5-nitroimidazole derivatives have been used in the treatment of trichomoniasis and other parasitic diseases such as amebiasis and giardiasis. However, studies show cases of resistance to the drug, causing new strategies to be taken to develop new therapeutic targets (Kirkcaldy et al., 2012; Upcrof et al., 2009; Schwebke and Barrientes, 2006).

Several natural products have been tested against various pathogens due to the large amount of secondary metabolites present in plants, which serve as a defense line for both the vegetable and pharmaceutical applications for some diseases. Green tea is amongst the most widely consumed beverages worldwide and is often touted for its wealth of medicinal effects (Moon et al., 2007; Khan and Mukhtar, 2008; Thielecke and Boschmann, 2009; Ahmed, 2010). Besides, green tea is produced from unfermented leaves, which have a large amount of antioxidants, which provides several health benefits and reduces the risk of diseases (Saito et al., 2009).

The flavonoids present in green tea are called catechins, corresponding to 30 to 40% of the solid components. The major catechin of green tea is called (-)-epigallocatechin-3-gallate (EGCG), corresponding to 59% of the total catechins. Studies have shown that green tea has a broad spectrum of activities, including anti-inflammatory, antioxidant, anticancer and antimicrobial properties, which are primarily exhibited by EGCG. Studies have reported that the effects of EGCG are linked to the inhibition of bacterial adherence in host cells (Reygaert, 2014). Thus, the objective of this work was to evaluate the ultrastructural alterations in *T. vaginalis* caused by the use of EGCG in different concentrations of treatment.

2. MATERIAL AND METHODS

2.1. Chemical

EGCG (Sigma-Aldrich Chemical Co. – St. Louis, MO) from green tea (stock solution 10 mM) was solubilised in sterile distilled water. The reagent was used immediately after preparation.

2.2. Parasite culture

The JT strain of *T. vaginalis* was isolated at the Hospital Universitário, Universidade Federal do Rio de Janeiro, Brazil, and has been maintained in culture for several years.

Trophozoites were cultivated in TYM Diamond's medium (Diamond, 1957) supplemented with 10% fetal bovine serum. The cells were grown for 24 h at 37º C.

2.3. In vitro antiproliferative activities

The parasites were cultured in TYM medium for 12 h at 37 °C (initial inoculum $1x10^4$ and $1x10^5$ parasites/mL). After this period, several concentrations of EGCG (10–500 µM) were added to the culture medium, and the parasites were incubated for up to 48 h at 37 °C. The number of parasites/mL was calculated after incubation (6–48 h) using a Neubauer haemocytometer. The viability of the parasites was checked trough the locomotion of the flagellum. Parasites treated with 5% sterile distilled water (maximum concentration of vehicle) were used as control.

2.4. Scanning electron microscopy (SEM)

Parasites were washed with PBS and fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The cells were then post-fixed for 15 min in 1 % OsO4, dehydrated in ethanol and critical point dried with liquid CO_2. The dried cells were coated with gold-palladium to a thickness of 15 nm and then observed with a Jeol JSM-5600LV scanning electron microscope (Tokyo, Japan).

2.5. Transmission electronic microscopy (TEM)

The parasites were washed with PBS and fixed in 2.5 % glutaraldehyde in 0.1-M cacodylate buffer, pH 7.2. The cells were then post-fixed for 30 min in 1 % OsO4, dehydrated in acetone and embedded in *Epoxy* Embedding Medium kit (Sigma-Aldrich). Ultra-thin sections were harvested on 300-mesh copper grids, stained with 5 % uranyl acetate and 1 % lead citrate, and observed with Tecnai™ G2 Spirit BioTWIN transmission electron microscope.

2.6. HeLa cell viability assay

For the cell viability test, a 24-well plate was used. For each well, a final volume of 500 µL containing $1x10^5$ cells was incubated. Two controls were carried out: negative control (only HeLa) and vehicle (DMSO). The plates were maintained for 24 h in a 37 ºC oven with 5% CO_2

until reaching 80% confluency, monitored with the help of an inverted light microscope. Three independent experiments were performed in triplicate.

2.7. Cytotoxicity assays in HeLa

Parasite-host cell interactions were initiated as described above. At the end of the incubation periods, MTT (0.5 mg/mL in DMEM) was added to the remaining host cells and incubated for an additional 1 h at 37 ◦C. Afterwards, the medium was discarded, and 1 mL of an acid isopropanol solution (4 M HCl: isopropanol PA; 1:99, v/v) was added to each well to solubilize the colored formazan product that was formed. Absorbance was read at 570 nm, and the background was subtracted at 650 nm on GloMax®-Multi Microplate Multimode Reader (Promega). The viability was calculated with the following equation: 1 – (E/C). All measurements of experimental (E) samples (A570 nm-650 nm) were indexed to those of control (C) samples (E/C), which showed no loss of viability, and then subtracted from 1.0. All data points were performed in triplicate. The results are the average of three independent experiments.

2.8. Hemolytic activity

Human venous blood was used for the hemolysis test. The blood collected was from the authors of the research, one of the female gender and the other of the male gender, both with blood type O^+. Three independent experiments were performed in quadruplicate for each individual. A 2% red blood cell suspension was prepared (2 mL homogenized blood collected with anticoagulant in 18 mL of 0.85% saline plus 10 mM $CaCl_2$ pH 7.2.) The suspension was then centrifuged at 2500 rpm for 2 min and was distributed in 96-well plate 75 µL of the final concentrations (0.1, 1.0 and 2.5 mg/mL) of the extract and 75 µL of the red blood cell suspension were added. The plate was incubated at 37 ºC for 3 h and then centrifuged at 3500 rpm for 4 min. Triton X-100 was used as the positive control, and the negative control was 0.85% saline. Microplate reader GloMax®-Multi Microplate Multimode Reader (Promega) at 540 nm.

2.9. Statistical analysis

The results for all assays are the average of three independent experiments performed at least in duplicate. Statistical comparison was performed using ANOVA test and using

computer analysis (GraphPad Prism v. 5.00, California, USA). $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of EGCG on growth of *T. vaginalis*

Initially, we evaluated the effects of EGCG on *T. vaginalis* growth. Treatment with EGCG decreased the culture growth and provoked a trichomonacidal effect without dose-dependent behavior. *T. vaginalis* growth was only restored, but at a lower rate when compared to control, when parasites were treated with 10 and 500 μM EGCG for up to 6 and 12 h, for both concentrations. Based on these results, doses of 10 and 500 μM EGCG for 12 h were selected for the subsequent experiments (Figure 1).

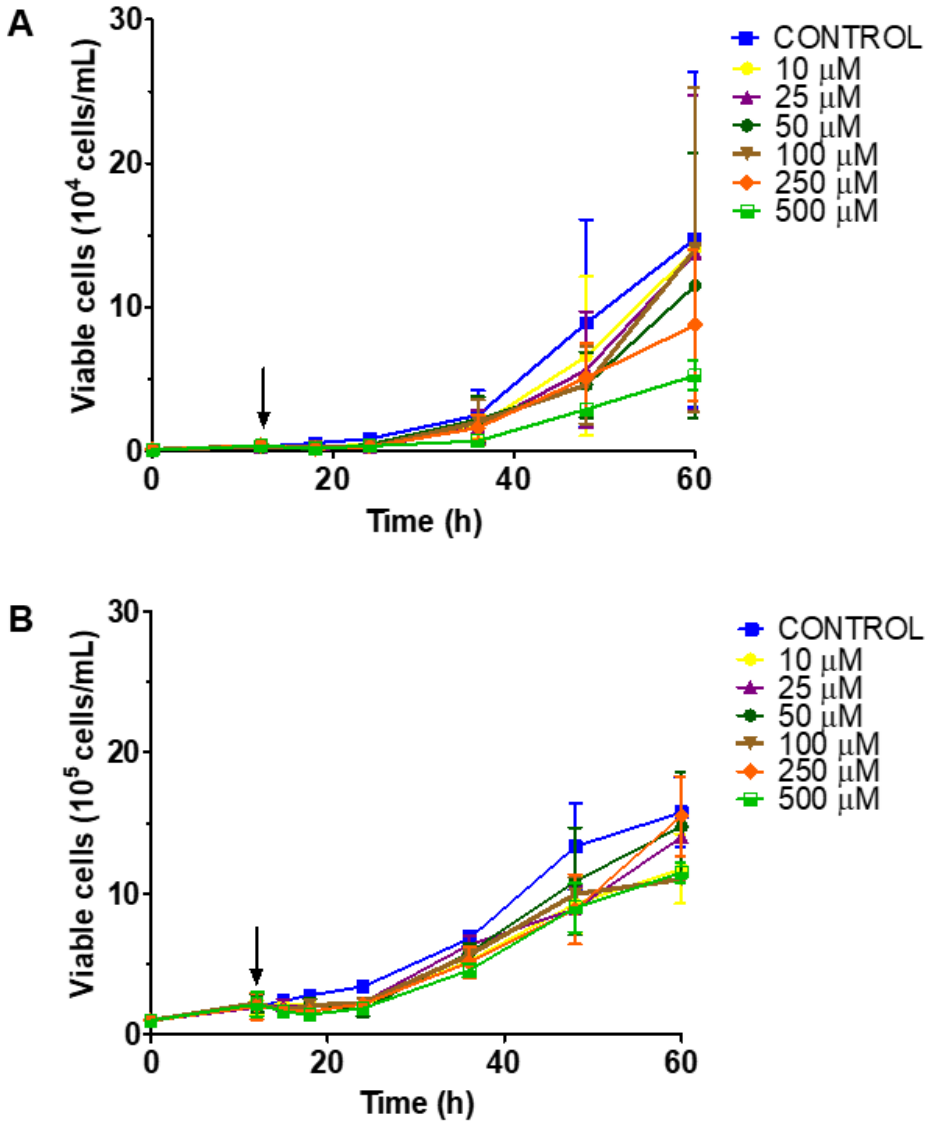

Figure 1. Growth curve of *T. vaginalis* strain JT under effect of different concentrations of EGCG at 10^4 cells/mL (A) and 10^5 cells/mL (B). The parasites were initially cultured for 12 h at 37 °C (initial inoculum: $1x10^4$ (A) and $1x10^5$ (B) parasites/mL). After this period (arrow), 10 – 500 µM of EGCG were added to the culture medium and the parasites were incubated for up to 48 h at 37 °C. The cell growth was calculated after 6 to 48 h of incubation. Parasites incubated with 5 % water were used as a control. Values are expressed as the means ± SD in three independent experiments, each performed in triplicate.

3.2. Effects of EGCG on ultrastructure of *T. vaginalis*

To determine the effects of EGCG on the morphology and fine structure of *T. vaginalis*, parasites treated with 10 or 500 µM of compound for 6 h and 12 h were observed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Untreated *T. vaginalis*, grown in axenic medium, is characterised by a pear-shaped body, four anterior

flagella, an undulating membrane reaching the posterior end of the cell body and a recurrent flagellum continuing beyond the undulating membrane by a free-trailing portion that is apparent using SEM (Figure 2a). By TEM, one anterior ellipsoid nucleus, a pelta-axostyle complex, a well-developed Golgi complex, hydrogenosomes with double membrane and an endoplasmic reticulum sparsely found around the nucleus and in close association with the hydrogenosomes are observed in the parasite (Figure 2b).

The EGCG treatment induced the transformation of *T. vaginalis* into pseudocystic (Figure 3D) and provoked the appearance of several morphological alterations indicative of cell death, including (a) plasma membrane blebbing (Figure 3A, B, D), (b) wrinkled cells (Figure 3A), (c) concentric membrane whorls, which resembled autophagic vacuoles (Figure 4A-C), (d) hydrogenosomes with double membrane damage (Figures 4A-C), (e) intense cytosolic vacuolisation (Figure 4A-C) and (f) plasma membrane disruption and lysed cells (Figure 4C). Ultrastructural changes in the nucleus, such as chromatin condensation and vacuolisation, were also observed in 10-μM EGCG-treated parasites (Figure 4A).

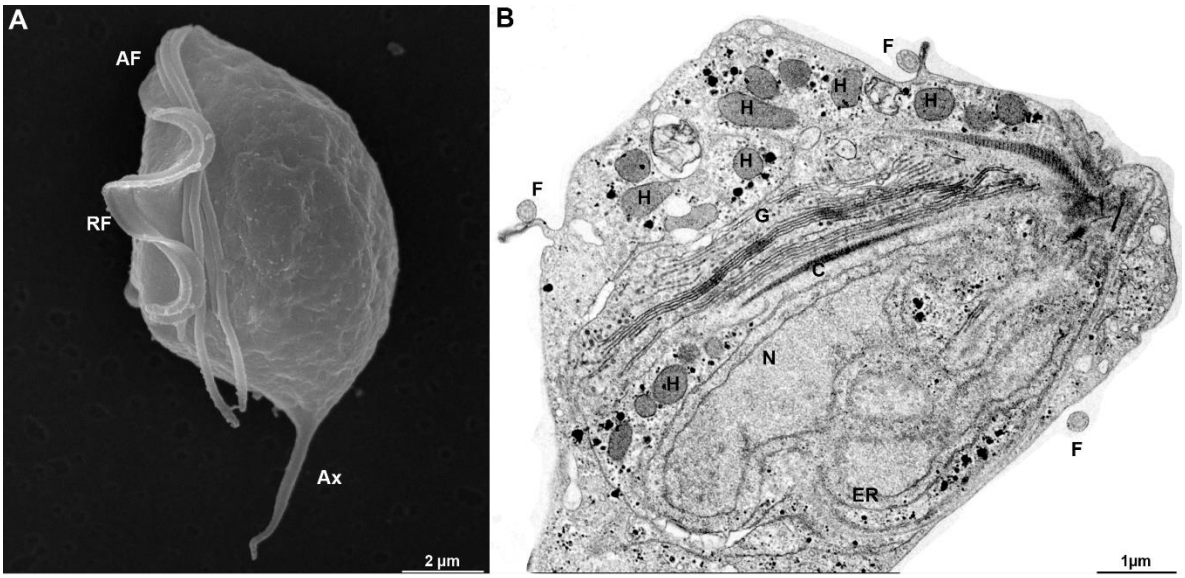

Figure 2. Fine structure of untreated *T. vaginalis*. (A) SEM. Parasite exhibits four anterior flagella (AF) and one recurrent flagellum (RF). The axostyle (Ax) tip is visible. (B) TEM of a longitudinal section of the parasite. The parasite displays one anterior nucleus (N), Nucleolus (Nu), Golgi complex (G), hydrogenosomes (H), and endoplasmic reticulum (ER). Bars at A 2 μm and B 1 μm.

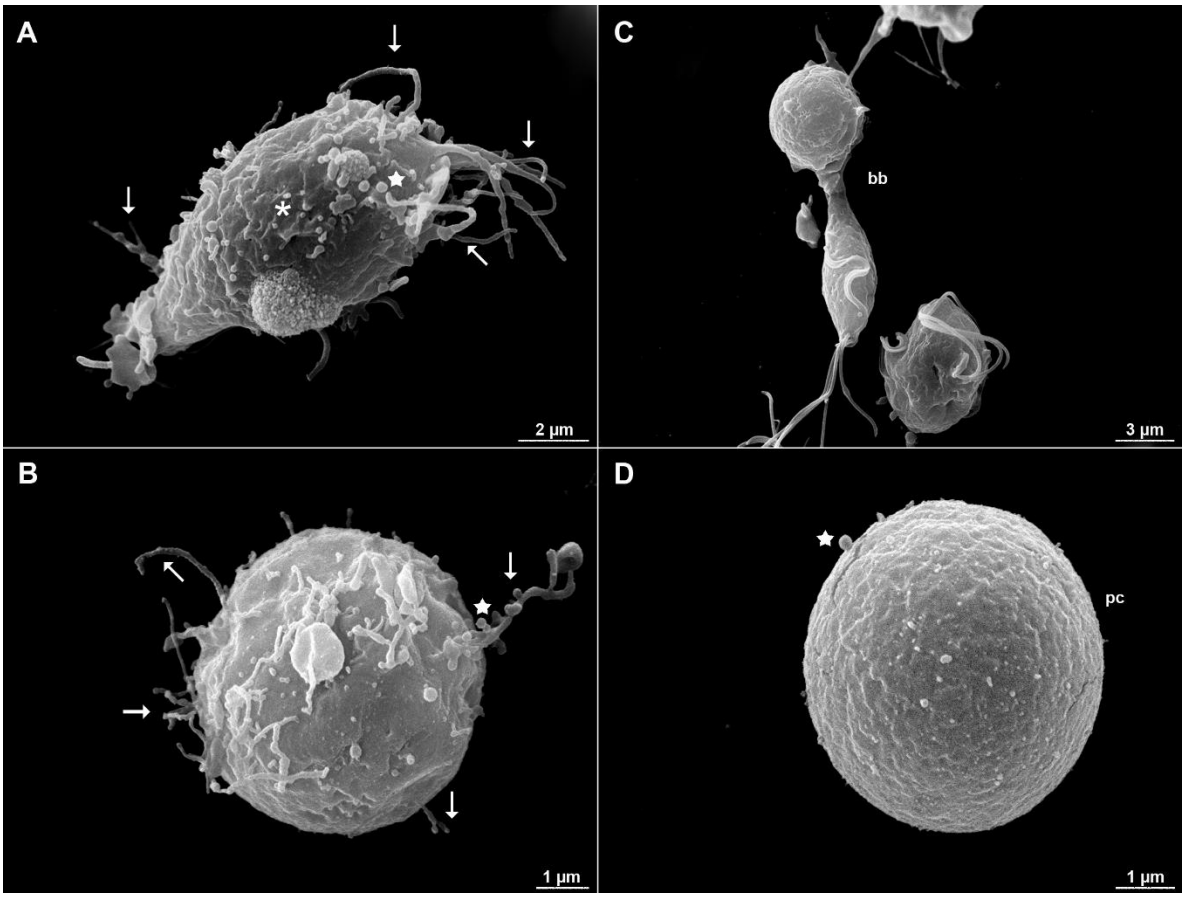

Figure 3. SEM of EGCG-treated *T. vaginalis*. Parasites were incubated with 10 µM (A, B) (A and B) and 500 µM (C, D) EGCG for 6 h and 12 h, respectively. Some parasites exhibit morphological alterations indicative of cell death, such as appearance of wrinkled cells (asterisk), membrane projections (arrows) and membrane shedding (stars). Other changes such as budding (bb) and pseudocyst (pc) can be observed. Bars at B, D 1 µm, A 2 µm and C 3 µm.

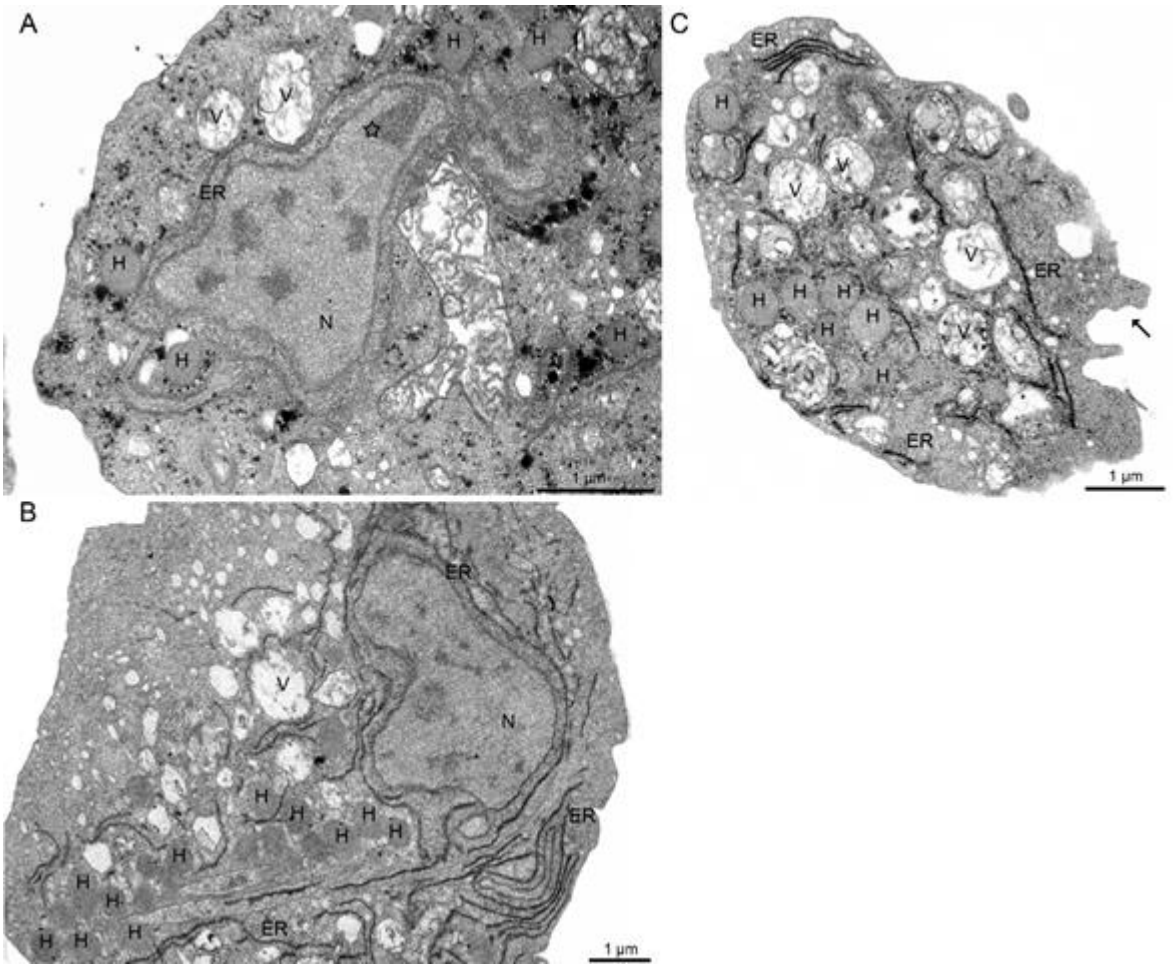

Figure 4. TEM of EGCG-treated *T. vaginalis*. Parasites were incubated with 10 µM (A, B) (A and B) and 500 µM (C) EGCG for 6 h and 12 h, respectively. The parasites exhibit alterations indicative of cell death, such as endoplasmic reticulum expansion (ER), autophagic vacuoles (v), hydrogenosomes with double membrane damage (H), membrane disruption (arrow) and cell lysis (asterisks). Bars at A, B, C 1 µm, 2 µm.

After treatment with EGCG at 10 µM and 500 µM, SEM analysis showed that changes were most evident at 500 µM. For both concentrations, more than half of the cells are in their typical form. The number of cells at 500 mM was twice that of 10 mM. (Figure 5). At least 500 Trichomonas were counted through SEM for each concentration tested at 6 and 12 h times to quantify those that were in cell division. It was found that less than 20% of *Trichomonas* were in cell division (Figure 6).

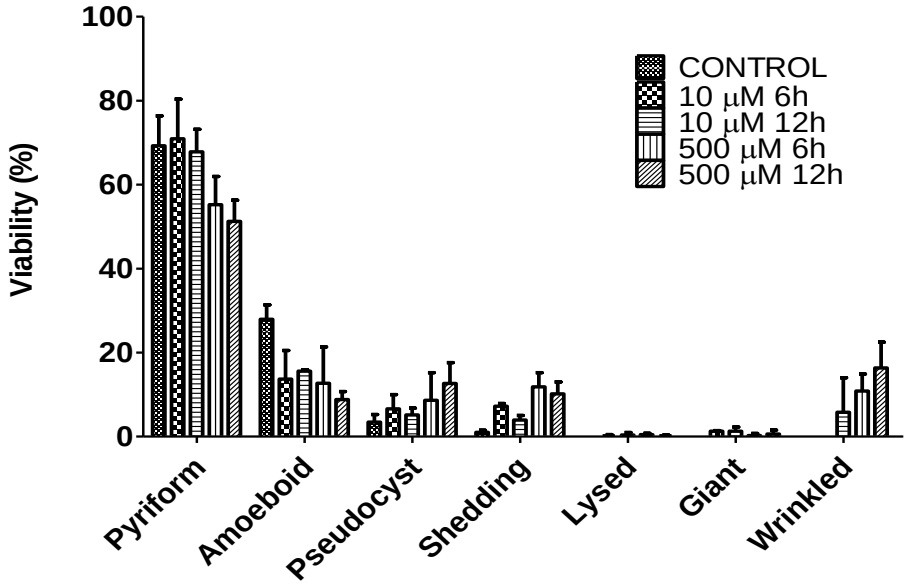

Figure 5. Quantitative analysis of cellular modifications from SEM treated with EGCG at 10 µM and 500 µM for 6 h and 12 h. Three independent experiments were performed in triplicate, with a minimum count of 500 cells. Bars are expressed as means ± SD.

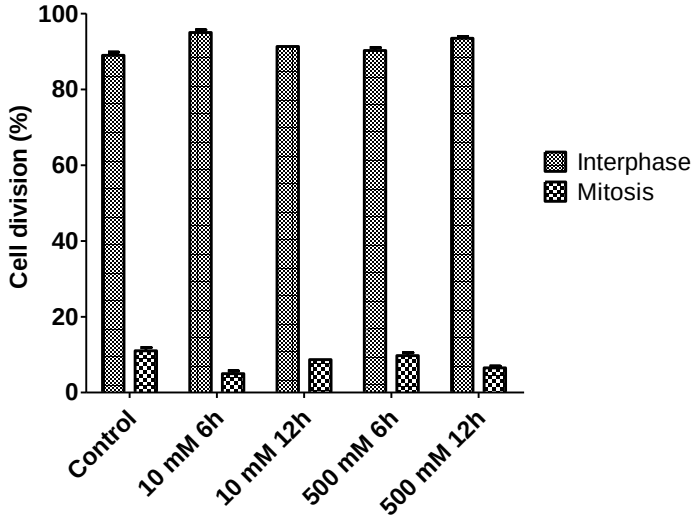

Figure 6. Quantification of *T. vaginalis* through SEM to identify the percentage of trophozoites in cell division after 6 and 12 h of treatment with EGCG at concentrations of 10 µM and 500 µM. Bars are expressed as means ± SD.

3.3. Cytotoxicity assays in HeLa

Cytotoxicity assays were performed to evaluate the effects of the EGCG on HeLa cells. MTT assays showed that at the concentration of 0.5 µM, HeLa cells did not have the viability affected. At concentrations of 1 µM and 5 µM, cytotoxicity of 35,95% and 99,17% was detected, respectively (Figure 7).

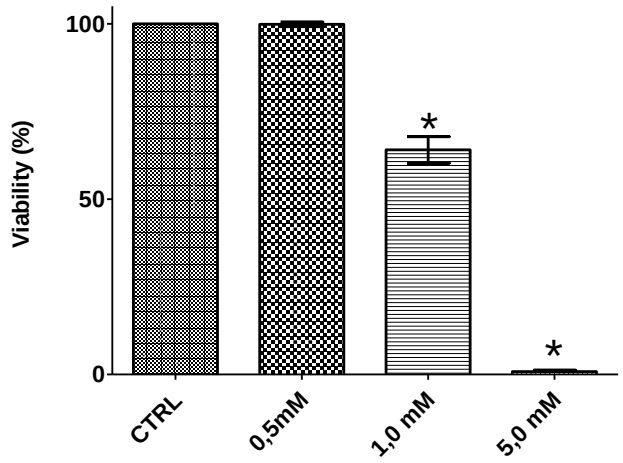

Figure 7. MTT viability assay of EGCG. Results are expressed as the mean ± SD of three independent experiments that were each performed in triplicate. *P<0.001 compared to control.

3.4. Hemolytic activity of EGCG

The hemolytic activity of EGCG was evaluated, and after 3 h of incubation, hemolysis was not observed in none concentrations tested.

4. Discussion

Epigallocatechin-3-gallate (EGCG) is the most abundant and widely studied flavonoid from green tea catechins. EGCG desperted considerable interest as a pharmaceutical compound with several therapeutic activities (Khan et al., 2006). In the present study, we demonstrate that EGCG exerts its effect on *T. vaginalis* inducing ultrastructural changes on morphology of this parasite. Treatment of trophozoites with EGCG resulted in no a time- and dose-dependent inhibition on cellular proliferation. Incubation with EGCG significantly inhibited *L. amazonensis* trophozoites proliferation after 6 h and 12 h. These results demonstrate the activity of EGCG against *T. vaginalis*.

Ultrastructural alteration of the mitochondria was observed in promastigotes treated with EGCG. Therefore, EGCG exerts its antileishmanial effect on *L. amazonensis* promastigotes by affecting parasite mitochondrial function (Inacio et al;. 2012). Our results showed EGCG effects on the ultrastructural changes on *T. vaginalis*, mainly in hidrogenosomes. Mitochondria are responsible for respiration and oxidative phosphorylation in eukaryotes and they provide ATP through respiratory-coupled oxidative phosphorylation.

During oxidative phosphorylation, electrons are moved thorough the mitochondrial respiratory chain, and a proton gradient is established across the inner mitochondrial membrane as the energy source for ATP production (Affranchino et al., 1985).

Hydrogenosomes and mitochondria presents similarities each other, such as: (1) both are surrounded by two membranes and present a granular matrix (Benchimol and De Souza, 1983); (2) both produce ATP (Lindmark and Müller, 1973); (3) both participate in the metabolism of pyruvate formed during glycolysis (Lindmark and Müller, 1973); (4) they are able to utilize oxygen as a terminal electron acceptor (Cerkasov et al., 1978); (5) both present a relationship with the endoplasmic reticulum (Benchimol, 2008a) and (6) they incorporate calcium (Benchimol and De Souza, 1983; Chapman et al., 1985).

Concerning inhibition of growth in protists, a study with *Trypanosoma brucei* showed that in the presence of a phosphatase inhibitor, EGCG reduced acetyl-CoA carboxylase (ACC) activity, whereas in the absence of this inhibitor, no inhibition of activity of ACC (Vigueira et al., 2012). Our results showed that there is a marked decrease in the number of trophozoites of *T. vaginalis* in two times in the growth curve, 6h and 12h. Through the TEM, it can be observed that the hydrogensomes were electron-lucent, possibly indicating that they were being affected by EGCG.

The metabolism of metabolic pyruvate occurs in the hydrogensomes of *T. vaginalis*. The process of pyruvate metabolism begins in the cytosol, where glycolysis generates intermediates, such as pyruvate and malate, that enter the hydrogensome. The pyruvate is decarboxylated by oxidation, and the electrons are transferred to protons, with the formation of H_2. ATP is generated by the *T. vaginalis* hydrogenosome via substrate-level phosphorylation involving acetyl CoA released by the decarboxylation of pyruvate (Benchimol, Pereira-Neves and Souza, 2016).

EGCG has been demonstrated to inhibit fatty acid synthesis (Wang and Tian, 2001; Brusselmans et al., 2003) through its effect on the regulation of acetyl-CoA carboxylase (ACC) (Huang et al., 2009). ACC catalyzes the first step in fatty acid synthesis the ATP-dependent carboxylation of acetyl-CoA (Tong and Harwood, 2006). ACC is negatively regulated by phosphorylation by a key regulator of cellular energy metabolism, AMP-activated protein kinase (AMPK) (Barber et al., 2005; Brownsey et al., 2006). EGCG treatment activates AMPK, leading to increased phosphorylation of human ACC, which resulted in its inhibition (Moon et al., 2007; Huang et al., 2009).

In our study, we also observed the presence of nucleoid and autophage vacuoles in hydrogenosomes of *T. vaginalis* treated with EGCG. Reports with *T. foetus* under serum deprivation, drug treatment (Madeiro and Benchimol, 2004; Benchimol et al., 1996a; Ribeiro et al., 2002a) and also under normal conditions presents signs of cell death such as apoptosis (Mariante et al., 2003, 2006) and autophagy (Benchimol, 1999). An inactive hydrogenosome can be promptly recognized by a dense deposit in its matrix, known in early literature as nucleoid. Autophagy occurs in cells where hydrogenosomes or other cell structures are old, or need to be removed. In autophagy, initialy are observed is the cisternae of the rough endoplasmic reticulum surrounding and enclosing the altered hydrogenosome, forming an isolation membrane, and after, an autophagic vacuole. Lysosomes fuse with the autophagic vacuole forming a degradative structure, the autophagosome. Hydrogenosomes are thus degraded, as occurs in some drug treatments (Benchimol, 1999, Benchimol, 2009).

Reports suggest that EGCG showed cancer–preventive, antioxidant, antimutagenic, apoptotic, and anti-inflammatory properties (Shankar et al., 2007; Hsu and Liou, 2011; Zhu et al., 2011). Our results showed that EGCG treatment decreased the cell viability in a dose dependent in 24 h (Figure 1). Others studies also showed that EGCG inhibits cancer cell viability in a dose dependent manner in various cancer cells (cervical, pancreatic, hepatic carcinoma, ovarian, colon and T-cell leukemia (Takada et al., 2002; Harakeh et al., 2008; Qiao et al., 2009; Shirakami et al., 2009; Zhou et al., 2011).

Sharma et al. (2012) observed that untreated HeLa cells showed no signs of apoptosis, exhibiting large and prominent nuclei. However, ECGC treatment resulted in the accumulation of apoptotic changes such as nuclear condensation, chromatin fragmentation, and the formation of nuclear debris identified as apoptotic bodies in these cells that increased in a time-dependent manner. Other studies have established that chemopreventive agents exert their effects on cancer cells through apoptosis (Gupta et al., 2011; Lee et al., 2008; Sharma et al., 2006; D'Agostini et al., 2005).

5. CONCLUSION

This study shows that the EGCG shows inhibits and causes ultrastructural changes on the morphology in *T. vaginalis*. Our results further contribute to a better understanding of the role of EGCG in hydrogenosomes of *T. vaginalis*. However, more specific studies such as flow

cytometry to test for the labeling of hydrogens are affected to prove the real effect of the compound in these organelles.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Funding Statement

This work was suppoted by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Instituto Aggeu Magalhães (IAM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We thank to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for funding research and Instituto Aggeu Magalhães for the place assigned to carry out the research (IAM).

Data Availability

All relevant data are within the paper and its Supporting Information files.

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4 CONCLUSÃO

Os resultados apresentados nesta tese permitem considerar que o extrato bruto das raízes de *B. floribunda* metanol:água apresentou atividade anti-*T. vaginalis in vitro* de maneira dose-dependente em 24 horas de tratamento. O EGCG apresenta atividade contra *T. vaginalis in vitro*, no entanto não há relação dose-dependente em concentrações de 10^5 células.

Os compostos BFRMA e EGCG apresentaram alta citotoxicidade em linhagem celular HeLa. EGCG mostrou intensa alteração nas membranas dos hidrogenossomos, no entanto mais testes com aplicação de outras técnicas, como citometria de fluxo poderiam confirmar esse resultado.

Nos testes de atividade hemolítica, EGCG não apresentou hemólise nas três concentrações testadas (0,1, 1,0 e 5,0 µM), enquanto que BRFMA em concentrações mais altas (1,0 e 2,5 mg/mL) apresentaram este efeito.

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ANEXO A – NORMAS PARA SUBMISSÃO DE ARTIGO NA REVISTA JOURNAL OF PARASITOLOGY RESEARCH

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A clinical study presents the methodology and results of a study that was performed within a clinical setting. These studies include both clinical trials and retrospective analyses of a body of existing cases. In all cases, clinical studies should include a

description of the patient group that was involved, along with a thorough explanation of the methodology used in the study and the results that were obtained.

When publishing clinical trials, Hindawi aims to comply with the recommendations of the International Committee of Medical Journal Editors (ICMJE) on trial registration. Therefore, authors are requested to register the clinical trial presented in the manuscript in a public trial registry and include the trial registration number at the end of the abstract. Trials initiated after July 1, 2005, must be registered prospectively before patient recruitment has begun. For trials initiated before July 1, 2005, the trial must be registered before submission.

Reviews

A review article provides an overview of the published literature in a particular subject area.

Formatting

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Title and Authorship Information

The following information should be included:

- Manuscript title
- Full author names
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Abstract

The manuscript should contain an abstract. The abstract should be self-contained, citation-free, and should not exceed 200 words.

Introduction

This section should be succinct, with no subheadings.

Materials and Methods

This part should contain sufficient detail that would enable all procedures to be repeated. It can be divided into subsections if several methods are described.

Results and Discussion

This section may be divided into subsections or may be combined.

Main Text (Review only)

This section may be divided into subsections or may be combined.

Conclusions

This should clearly explain the main conclusions of the article, highlighting its importance and relevance.

Data Availability (excluding Review articles)

This section should describe how readers may access the data underlying the findings of the study.

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Authors must declare all relevant interests that could be perceived as conflicting. Authors should explain why each interest may represent a conflict. If no conflicts exist, the authors should state this. Submitting authors are responsible for coauthors declaring their interests.

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Tables should be cited consecutively in the text. Every table must have a descriptive title and if numerical measurements are given, the units should be included in the column heading. Vertical rules should not be used.

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We provide some illustrative examples of “Data Availability” statements below. This list is not exhaustive, and authors may find that they require a statement different from the samples listed here:

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In the reference list: [1] A. N. Author, B. N. Author, C. N. Author et al., “Dataset title,” Dryad Digital Repository, Doi:10.5061/dryad.xxxxx, 2016.

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ANEXO B – NORMAS PARA SUBMISSÃO DE ARTIGO NA REVISTA PARASITOLOGY RESEARCH

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• Contributed new methods or models
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The e-mail address, and telephone number(s) of the corresponding author
If available, the 16-digit ORCID of the author(s)
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REFERENCES

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Negotiation research spans many disciplines (Thompson 1990).

This result was later contradicted by Becker and Seligman (1996).

This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

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Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

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Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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