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ERWELLY BARROS DE OLIVEIRA

AVALIAÇÃO DAS ATIVIDADES BIOLÓGICAS DE COMPOSTOS FENÓLICOS: NATURAIS (CUMARINA) E DERIVADOS COMERCIAIS (3-HIDROXICUMARINA E 4-HIDROXICUMARINA)

Recife

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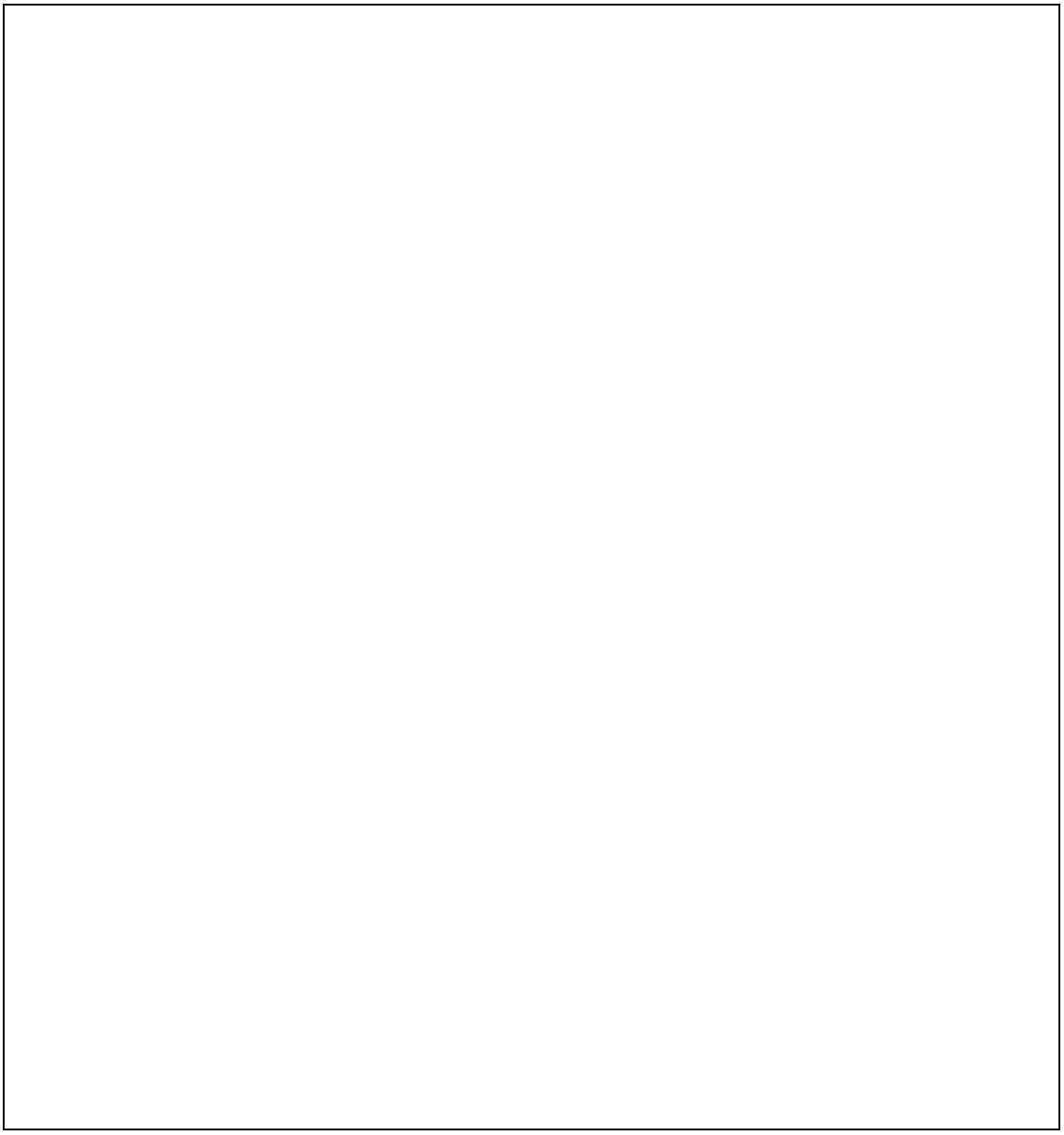

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RESUMO

OLIVEIRA, E.B. Avaliação das atividades biológicas de compostos fenólicos: naturais (cumarina) e derivados comerciais (3-hidroxicumarina e 4-hidroxicumarina). 2016. 122f. Dissertação (Mestrado). Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.

As plantas usadas na medicina popular têm sido alvo constante pela indústria farmacêutica na busca de novos protótipos úteis para a fabricação de fármacos direcionados ao tratamento de variadas doenças. Isso tem levado ao ressurgimento do interesse por metabólitos secundários produzidos por vegetais como os compostos fenólicos, dentre os quais ressaltam as cumarinas. Essas substâncias purificadas exibem atividades biológicas potentes e relevantes, além de apresentarem baixa toxicidade nos mamíferos. Esse conjunto de benefícios mantém as cumarinas como alvo de investigação nas pesquisas atuais e fomenta o interesse farmacêutico a nível mundial. O objetivo deste trabalho foi estudar as atividades biológicas de compostos fenólicos: cumarina (1,2-benzopirona) e derivados comerciais (3-hidroxicumarina e 4-hidroxicumarina). As formas promastigotas de *Leishmania (L.) amazonensis* (10^5 parasitas/mL) foram testadas nas concentrações de 1,56 a 400 µg/mL para obtenção da IC_{50} através do método colorimétrico do MTT. A anfotericina B foi utilizada como controle positivo e o DMSO como controle negativo. A morfologia das formas promastigotas da *L.(L.) amazonensis*, sob efeito da 3-hidroxicumarina, foi analisada através da microscopia eletrônica de varredura, em função da melhor atividade leishmanicida (IC_{50} = 6,25 µg/mL), quando comparada a cumarina e a 4-hidroxicumarina (200 µg/mL e 400 µg/mL, respectivamente). A citotoxicidade foi realizada em macrófagos de linhagem J774 ($2x10^5$ células/mL), células Vero ($1x10^5$ células/mL) e células HeLa ($2x10^5$ células/mL) sob efeito da 3-hidroxicumarina nas concentrações de 0,78 a 400 µg/mL. A atividade citotóxica das três linhagens de células foi estatisticamente significativa para as maiores concentrações de 100 a 400 µg/mL (p<0,05) e o estudo morfológico revelou alterações como decréscimo da densidade celular, com presença de células arredondadas ou retraídas. Os compostos testados mostraram um padrão concentração-dependente em relação as atividade antioxidante (DPPH) e hemolítica. Os resultados obtidos neste trabalho colocam os compostos fenólicos (cumarina, 3-hidroxicumarina e 4-hidroxicumarina) em evidência para investigações futuras visando o desenvolvimento de novos agentes terapêuticos contra leishmanioses.

Palavras-chave: Atividades biológicas. Compostos fenólicos. Cumarinas. Hidroxicumarinas.

ABSTRACT

OLIVEIRA, E.B. Evaluation of biological activity of phenolic compounds: natural (coumarin) and trading derivatives (3-hydroxycoumarin and 4-hydroxycoumarin). 2015. 122f. Dissertation (Master). Federal University of Pernambuco, Recife, Pernambuco, Brazil.

The plant used in folk medicine have been targeted by the pharmaceutical industry constantly in search of new prototypes useful for the manufacture of drugs directed to the treatment of various diseases. This has led to a resurgence of interest in secondary metabolites produced by plants as phenolic compounds, among which highlight the coumarin. These purified substances exhibit potent and relevant biological activities, besides having low toxicity in mammals. This set of benefits keeps coumarins as research target on current research and promotes pharmaceutical interest worldwide. The objective of this work is to study the biological activity of phenolic compounds: coumarin (1,2-benzopyrone) and commercial derivatives (3-hydroxycoumarin and 4-hydroxycoumarin). *Leishmania (Leishmania) amazonensis* promastigotes (10^5 parasites/mL) were treated with 1.56 to 400 µg/mL to obtain IC50 values using the MTT bioassay. Amphotericin B was used as a control drug (0.19 to 100 µg/mL) and LIT-DMSO, negative control. The morphological changes of promastigotes *L. (L.) amazonensis* were analysed in scanning electron microscopy, in the best activity antileishmanial (IC_{50} = 6,25 µg/mL) when compared with coumarin and 4-hydroxycoumarin (200 µg/mL e 400 µg/mL, respectively). Cytotoxicity was determined using the MTT colorimetric method. The cells were added at a concentration of 1 x 10^5 cells / mL (Vero cells) and 2 x 10^5 cells / mL (HeLa cells and J774 macrophages) in 96 well plates where the 3-hydroxycoumarin was at concentrations from 0.78 to 400 µg / mL. The morphology of the cells was evaluated with the aid of an inverted phase contrast microscope and photographic records performed for each concentration tested. The 3-hydroxycoumarin was not cytotoxic to J774, Vero and Hela cells in lower concentrations and morphological changes in Vero, Hela and J774 cells were viewed from the concentration of 100 µg/mL of 3-hydroxycoumarin. The data of the antioxidant and hemolytic activities showed clearly that the data indicate the concentration-dependent activities of compounds. This study showed that coumarin and its derivatives (3-hydroxycoumarin and 4-hydroxycoumarin) can be considered in interesting candidate for future studies regarding as a prototype drug for the treatment of leishmaniasis, but more studies should be conducted to discovery of the possible mechanisms involved in the biological activities studied.

Keywords: Biological activities. Phenolic compounds. Coumarins. Hydroxycoumarins.

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LISTA DE ABREVIATUÇÕES E SIGLAS

OMS	Organização Mundial da Saúde
ANVISA	Agência Nacional de Vagilância Sanitária
LC	Leishmaniose Cutânea
UFPE	Universidade Federal de Pernambuco
LIKA	Laboratório de Imunopatologia Keizo Asami
UFPB	Universidade Federal da Paraíba
MTT	3-metil [4,5-dimetiltiazol-2-il]-2,5 difeniltetrazólio
DMSO	Dimetilsulfóxido
DMEM	*Dulbecco's modified Eagle's medium*
LIT	*Liver Infusion Tryptose*
IC_{50}	Concentração de inibição de 50% do crescimento em relação ao controle (parasitas)
CC_{50}	Concentração de inibição de 50% do crescimento em relação ao controle (células)
DPPH	2,2-difenil-1-picrilhidrazil
PBS	Solução tamponada com fosfato

SUMÁRIO

1. INTRODUÇÃO

O Brasil é um país de proporções continentais, cujo território ocupa mais da metade da América do Sul, com cerca de 8,5 milhões de km^2 chega a abranger diferentes zonas climáticas como o trópico úmido do Norte, o semiárido do Nordeste e as áreas temperadas no Sul (SPEKTOR, 2010). Em função dessas diferenças climáticas ocorrem consistentes variações ecológicas, em que se formam zonas biogeográficas distintas ou biomas. Neste contexto, o país é detentor da maior biodiversidade do mundo e segundo estimativas de Lewinsohn e Prado (2005) o número de espécies conhecidas no Brasil estaria entre 170 e 210 mil, sendo 103–134 espécies de animais e de 43-49 mil espécies de plantas. Esse imenso patrimônio possui uma considerável quantidade de produtos naturais a serem pesquisados, com um valor econômico-estratégico inestimável, despertando interesse nas mais variadas áreas de investigação como biológica, química, farmacêutica, médica, biomédica e econômica, entre tantas outras (CARLIXTO, 2003).

Os produtos naturais têm sido usados desde o primórdio da humanidade como fontes inesgotáveis de alimento, de matéria-prima para vestuários e habitações. É sabido que as grandes civilizações da antiguidade utilizavam as plantas para o tratamento de uma ampla variedade de doenças e as primeiras descrições sobre essas plantas medicinais foram realizadas pelo homem e referidas nas sagradas escrituras e nos papiros de Ebers, onde foram enumeradas mais de 100 doenças e a descrição da ação de um grande número de produtos de origem animais e vegetais (VILELA, 1977). No Brasil, as contribuições para o surgimento de uma medicina popular rica e original adveio dos escravos e imigrantes, além da assimilação dos conhecimentos, já existente, com a cultura dos indígenas (PHILLIPSON, 2001; PINTO et al., 2002).

Apesar de existir registros antigos que mostram o amplo uso das plantas como alternativa terapêutica, todo esse conhecimento era baseado na observação da natureza, na crença popular e no empirismo. No século XVIII, foi iniciado o isolamento das primeiras substâncias puras e esses recursos passaram a ser estudados como instrumentos científicos e os princípios ativos começaram a ser identificados e utilizados na medicina tradicional. Desde então, é crescente o interesse por estudos que possam elucidar os mecanismos relacionados à produção de substâncias bioativas, também conhecidas como metabólitos secundários, e a

utilização desses compostos como possíveis fármacos para diversas doenças (PHILLIPSON, 2001; PINTO et al., 2002).

Os metabólitos secundários têm um papel importante na adaptação das plantas aos seus ambientes e colaboram para que as mesmas possam ter uma boa interação com os diferentes ecossistemas. Esses compostos são pouco abundantes, não estão diretamente envolvidos com os processos vitais de biosíntese das plantas e representam uma fonte importante de substâncias farmacologicamente ativas (FUMAGALI et al., 2008; PEREIRA; CARDOSO, 2012). Os efeitos dessas substâncias bioativas podem variar consideravelmente dependendo de vários fatores como sazonalidade, ritmo circadiano, temperatura, disponibilidade hídrica, radiação ultravioleta, nutrientes, altitude, indução por estímulos mecânicos ou ataques de insetos (GOBBO-NETO; LOPES, 2007).

Os metabólitos secundários são classificados de acordo com a sua rota biosintética e segundo suas características químicas em nitrogenados, terpenóides e fenólicos (CORREA et al., 2008). Dentre os nitrogenados, os alcalóides possuem uma ampla atividade biológica reconhecida como antitumoral, antiplasmótica, antimicrobiana, antibacteriana, anticancerígena (HENRIQUE, 2010). Os terpenóides são conhecidos como defensivos de plantas e têm sido estudados para a melhor compreensão sobre a sua ação repelente, além de estarem presentes em óleos essênciais (JUNIOR, 2003; COLPO et al., 2014). Os fenólicos são utilizados como atrativos para a polinização ou dispersão de sementes e estruturalmente são substâncias que possuem pelo menos um anel aromático no qual um hidrogênio é substituído por um grupamento hidroxila, como os flavonóides, taninos, isoflavonóides e cumarinas (BUENO-SANCHEZ et al., 2009; SIMÕES et al., 2010).

Existem vários estudos com esses metabólitos secundários dirigidos a descoberta de suas propriedades farmacológicas, dentre os quais se destacam as cumarinas que possuem atividades como antitrombótica, anticancerígena, antimicrobiana e antitripanocida. Mais de 1.300 cumarinas tem sido identificada em plantas, bactérias e fungos, sendo encontradas principalmente nas famílias Asteraceae, Fabaceae, Oleaceae, Moraceae, Thymeleaceae, Apiaceae e Rutaceae (CZELUSNIAK et al., 2012). As cumarinas são relatadas em cerca de 150 espécies de diferentes plantas distribuídas ao longo de quase 30 famílias diferentes e são encontradas em todas as partes das plantas, com elevada concentração nos frutos (JAIN et al., 2013; REHMAN et al., 2013;VENUGOPALA et al., 2013).

As plantas usadas na medicina popular têm sido alvo constante pela indústria farmacêutica na busca de novos protótipos úteis para a fabricação de fármacos direcionados ao tratamento de variadas doenças. Isso tem levado ao ressurgimento do interesse por metabólitos secundários produzidos por vegetais como os compostos fenólicos, dentre os quais se ressaltam as cumarinas (WHO, 2003). Essas substâncias purificadas exibem atividades biológicas potentes e relevantes, além de apresentarem baixa toxicidade em mamíferos. Esse conjunto de benefícios mantém as cumarinas como alvo de investigação nas pesquisas atuais e fomenta o interesse farmacêutico a nível mundial (HOULT; PAYÁ, 1996).

Dentre as doenças que são pesquisadas novos fármacos, se destaca a leishmaniose. Essa doença é causada por protozoários do gênero *Leishmania* e transmitida pela picada de fêmeas de flebotomíneos. Ela está incluída pela Organização Mundial de Saúde (OMS) como uma das seis doenças endêmicas e negligenciadas de maior relevância no mundo (WHO, 2003). Apesar dessa grande prevalência, os medicamentos em uso na clínica estão longe de serem ideais, pois são de administração parenteral obrigatória em longo prazo, levam a quadros de elevada toxicidade com efeitos que incluem mialgia, arritmias cardíacas, hepatotoxicidade, alguns pacientes já mostram ausência de resposta ao tratamento e esse fato pode ser explicado pelo aumento da resistência dos parasitas (BRASIL, 2013).

Na ausência de vacinas, os fármacos permanecem como centro do controle dessa doença. Contudo, o tratamento é inadequado e se faz necessário desenvolver novos fármacos como terapias alternativas, sendo estes menos tóxico, de baixo custo, que proporcione melhores resultados e tenha uma boa aceitabilidade pelo paciente (TIUMAN et al., 2011).

2. OBJETIVOS

2.1. Objetivo geral

Estudar as atividades biológicas de compostos fenólicos: cumarina (1,2- benzopirona) e derivados comerciais (3-hidroxicumarina e 4-hidroxicumarina).

2.2. Objetivos específicos

2.2.1. Avaliar o efeito da 1,2-benzopirona, 3-hidroxicumarina, 4-hidroxicumarina sobre a forma promastigota da *Leishmania* (*Leishmania*) *amazonensis*;

2.2.2. Analisar as características morfológicas através de microscopia eletrônica de varredura das formas promastigotas da *Leishmania* (*Leishmania*) *amazonensis* sob ação das substâncias-teste;

2.2.3. Investigar a atividade citotóxica das substâncias-teste em macrófagos J774, célula Vero e célula cancerígena HeLa;

2.2.4. Observar as características morfológicas através de microscopia de contraste de fase das células de linhagens J774, Vero e HeLa sob ação das substâncias-teste;

2.2.5. Pesquisar a atividade antioxidante das substâncias-teste pelo método do sequestro do radical livre 2,2-difenil-1-picrilhidrazil (DPPH);

2.2.6. Determinar a atividade hemolítica das substâncias-teste.

3. REVISÃO DE LITERATURA

3.1. Plantas medicinais

3.1.1. Considerações gerais

A Organização Mundial da Saúde (OMS) define planta medicinal como "todo e qualquer vegetal que possui, em um ou mais órgãos, substâncias que podem ser precursoras de fármacos semi-sintéticos". As plantas têm sido usadas pelo homem há muito tempo na prática medicinal, principalmente sob a forma de recurso terapêutico, o que fez aumentar acentuadamente essa utilização nas últimas décadas. Dados da OMS mostram que aproximadamente 80% da população fez uso de algum tipo medicamento à base de produtos naturais para alívio de sintomatologia dolorosa ou desagradável (WHO, 1998).

Muitos fatores têm contribuído para esse grande índice do uso de plantas medicinais, entre eles, o alto custo dos medicamentos industrializados, o difícil acesso da população à assistência médica, além de ser a única forma de tratamento para as mais variadas doenças em cerca de 70 e 95% da população dos países em desenvolvimento (WHO, 2011; BADKE et al., 2012). Apesar dessa grande utilização, poucas são as informações que se encontram disponíveis sobre os seus constituintes, assim como sobre os riscos que essa prática traz à saúde humana (FONSECA; PEREIRA, 2004). Esse conhecimento empírico passou a ser estudado e levado em conta pela comunidade científica, despertando o interesse pela busca de novas moléculas bioativas para fins terapêuticos (VEIGAS JUNIOR et al., 2005).

Os estudos científicos têm sido realizados com a utilização de extratos, frações ou substâncias isoladas de plantas, com a finalidade de se avaliar a atividade biológica desses produtos, determinar os princípios ativos como metabólitos (primários e secundários); além de, investigar os mecanismos que podem proporcionar o surgimento desses princípios nas diferentes partes da planta (ARAÚJO et al., 2014).

Além disso, os produtos naturais têm provido a indústria farmacêutica uma das mais importantes fontes de compostos "modelos", uma vez que 40% das drogas atuais são derivadas de fontes naturais usando a própria substância ou a sua versão sintetizada (JASSIM; NAJI, 2003). Os medicamentos de origem vegetal representam claramente uma janela de

oportunidade na indústria de medicamentos estruturada por se tratar de um mercado poderoso à busca de novas moléculas para assegurar a competitividade na produção de novos medicamentos patenteados. E também representa a oportunidade de participar na elaboração de uma categoria de medicamentos denominada fitoterápicos no Brasil, que são extratos vegetais padronizados e validados do ponto de vista da sua eficácia, segurança e qualidade (BÔAS; GADELHA, 2007).

Segundo a Agência Nacional de Vigilância Sanitária, ANVISA, fitoterápico é todo o preparado (extrato, tintura, pomada, óleos essenciais, cápsulas, comprimidos, etc.) que utiliza como matéria-prima parte de plantas, como folhas, caules, raízes, flores, sementes, com eficácia e segurança validadas em estudos etnofarmacológicos, documentações tecnocientíficas ou ensaios clínicos (ANVISA, 2004).

3.1.2. Metabólitos secundários

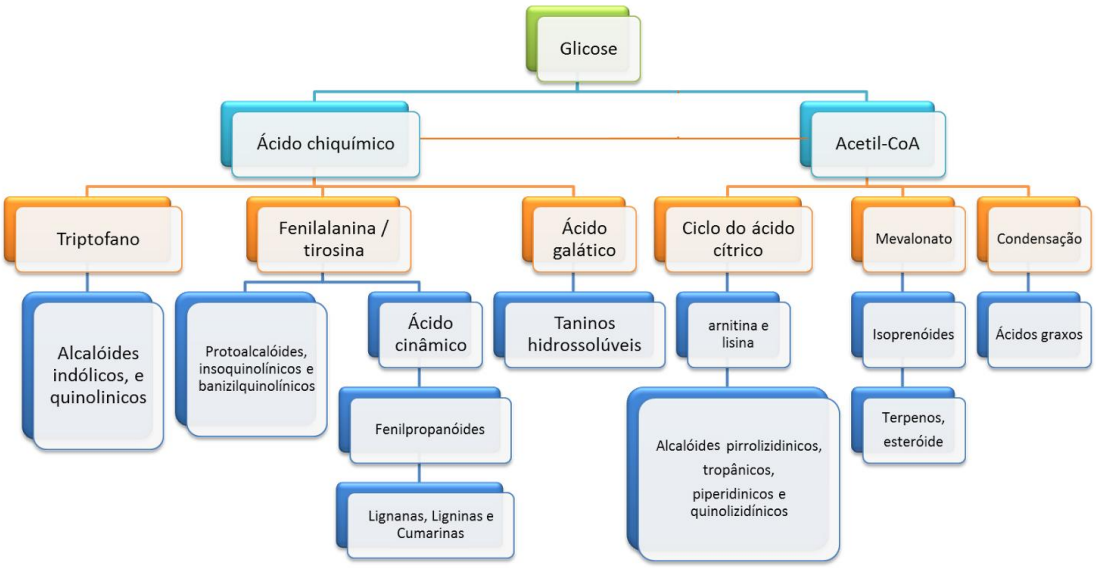
Os metabólitos secundários são originados a partir de duas rotas metabólicas derivadas da glicose: o ácido chiquímico e a do acetato (**Figura 1**). Esses metabólitos são encontrados em concentrações relativamente baixas e em determinadas plantas, apresentando-se, geralmente, com uma estrutura complexa, baixo peso molecular; além de possuir marcantes atividades biológicas. Embora não seja essencial para o organismo produtor, os metabólitos secundários são responsáveis pela sobrevivência e perpetuação da espécie, e podem ser influenciados por fatores externos tais como temperatura, radiação ultravioleta, nutrientes, altitude, indução por estímulos mecânicos ou ataques de insetos (GOBBO-NETO; LOPES, 2007; PEREIRA, 2011).

Dentre os metabólitos com atividades biológicas, destacam-se os compostos fenólicos. Essas substâncias são encontradas nas plantas sendo essenciais para o seu crescimento e reprodução, conferindo também alta resistência a microrganismos e pragas. Nos alimentos esses compostos são responsáveis pelo aroma, adstringência e coloração, influenciando no valor nutricional e na qualidade sensorial (PEREIRA, 2011; ROCHA et al, 2011).

Os fenólicos possuem estrutura variável englobando desde moléculas simples até moléculas com alto grau de polimerização e estão presentes nos vegetais na forma livre ou ligados a açúcares (glicosídios) e proteínas. Existem cerca de cinco mil fenóis, dentre eles,

destacam-se os flavonóides, ácidos fenólicos, fenóis simples, cumarinas, taninos, ligninas e tocoferóis (ANGELO; JORGE, 2007).

Figura 1- Ciclo de biossíntese dos metabólitos secundários.



Fonte: Adaptado de Simões, 2010.

Várias atividades farmacológicas são estudadas para esse grupo de compostos, entretanto os estudos mais promissores podem ser citados aqueles relacionados à atividade antiprotozoária *in vitro*. Estudos prévios mostram que essas substâncias são promissoras contra diferentes espécies de *Leishmania* e possuem potencial efeito contra a forma promastigota e amastigota (PAULA-JUNIOR et al., 2006; NAPOLITANO et al., 2003).

3.1.3. Leishmaniose

Leishmaniose é considerada como uma das doenças infecto-parasitárias endêmicas de grande relevância e um grande problema de saúde pública. De acordo com um recente relatório omitido pela OMS, há três formas de leishmanioses- visceral (muitas vezes conhecida como calazar e a forma mais grave da doença), cutânea (a mais comum) e a mucocutânea (se expressa por lesões destrutivas localizadas nas mucosas de vias áreas superiores), afetam coletivamente 12 milhões de pessoas em 98 países, com mais de 350

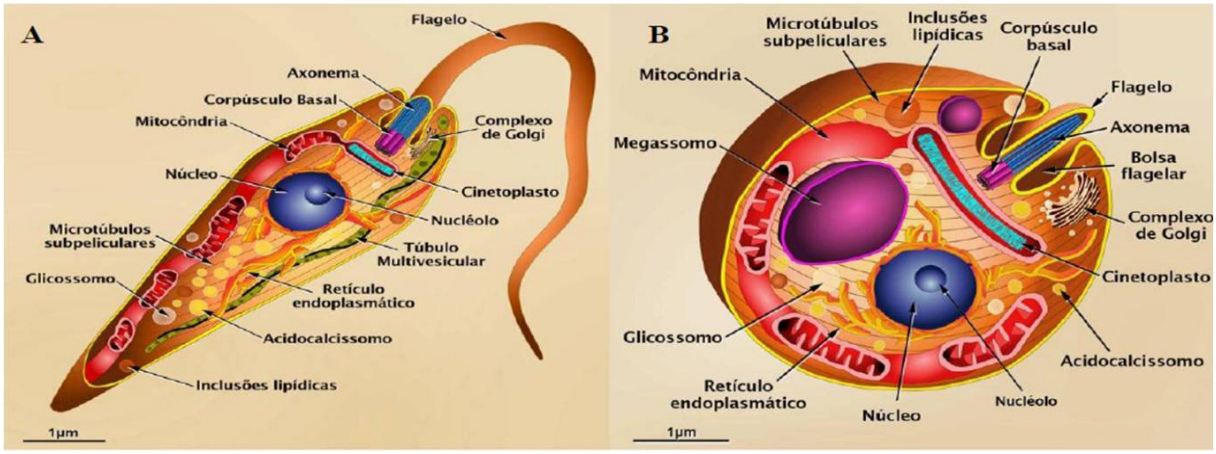
milhões delas em risco de contaminação. Além disso, existem cerca de 1,3 milhões de novos casos e 20 000 a 30 000 mortes ocorrem anualmente (WHO, 2010; WHO, 2015).

Leishmaniose cutânea (LC) é a forma mais comum da doença, provoca lesões cutâneas (úlceras) localizadas em áreas expostas da pele, com formato arredondado ou ovalado e costumam deixar cicatrizes atróficas (BRASIL, 2013). A LC tem ampla distribuição mundial e cerca de 75% dos casos ocorrem no Continente Americano (há registro de casos desde o extremo sul dos Estados Unidos até o norte da Argentina), Bacia do Mediterrâneo, Oriente Médio e Ásia Central. O Brasil é um dos países que registram o maior número de casos dessa doença (ALVAR et al., 2012; NAGLE et al., 2014).

No Brasil, a leishmaniose é transmitida através da picada de fêmeas de flebotomíneos infectados, pertencente à ordem Diptera, família *Psychodidae*, sub-família Phlebotominae, gênero *Lutzomyia*, conhecido popularmente no Brasil como mosquito-palha, birigui, cangalha, orelha-de-veado e existem sete espécies que são responsáveis pela leishmaniose tegumentar americana humana. São elas a *Leishmania (Viannia) brasilienses* (Leishmaniose Mucosa), *Leishmania (Viannia) guyanensis, Leishmania (Viannia) naiffi, Leishmania (Viannia) shawi, Leishmania (Viannia) lainsoni, Leishmania (Leishmania) amazonensis, L. (Viannia) lindenberg* (GUEDES et al., 2008; BRASIL, 2013).

A *Leishmania* é um protozoário pertencente à família Trypanosomatidae, parasito intracelular obrigatório das células do sistema fagocítico mononuclear, com duas formas principais: uma flagelada ou promastigota, encontrada no tubo digestivo livre ou aderida à parede do epitélio intestinal do inseto vetor, e outra aflagelada ou amastigota, observada nos tecidos dos hospedeiros vertebrados, no interior de um vacúolo parasitóforo das células do sistema mononuclear fagocitário (**Figura 2**) (ALCOLEA et al., 2010; BRASIL, 2013).

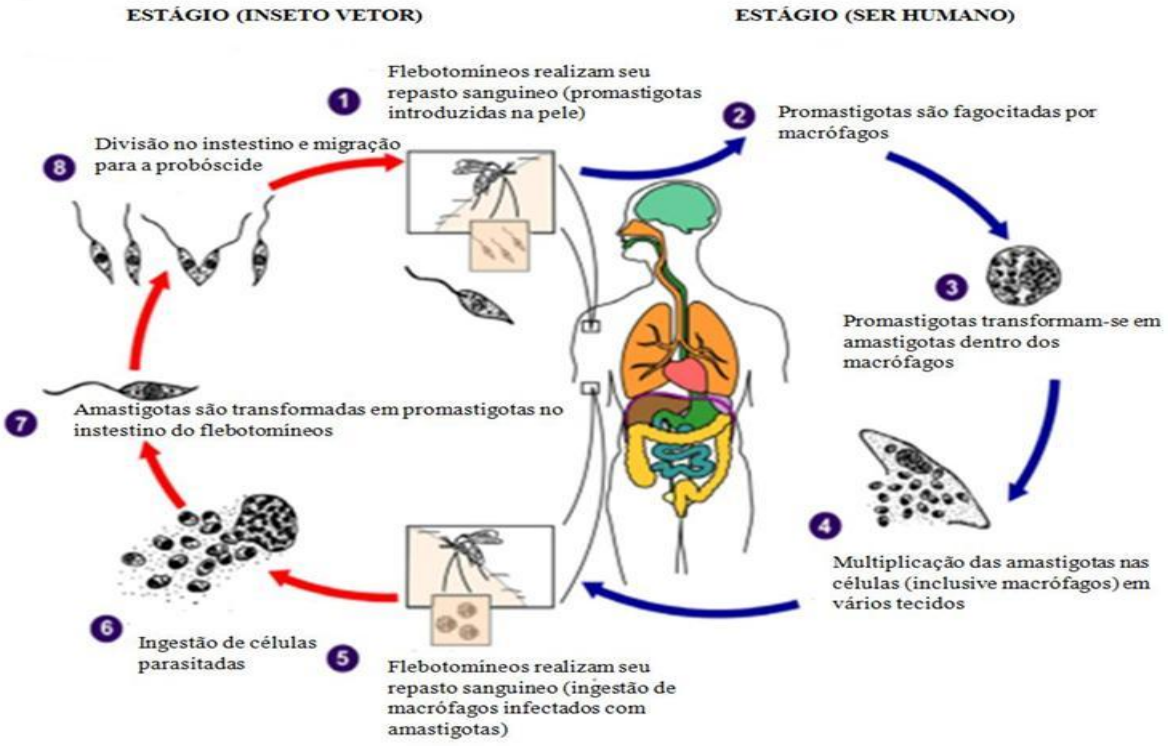
Figura 2- Formas evolutivas do parasita *Leishmania*.



Legenda: (A) Forma promastigota (B) Forma amastigota.
Fonte: Adaptado de TEXEIRA et al., 2013.

O ciclo de transmissão de leishmaniose varia de acordo com a região geográfica, espécie de parasitas, vetores, reservatórios e hospedeiros. O flebotomíneo infectado ao realizar o seu repasto sanguíneo introduz na pele do hospedeiro vertebrado as formas promastigotas metacíclicas. Esses parasitas invadem as células do sistema fagocitário mononuclear (principalmente macrófagos) através de vacúolos parasitóforos. Após a sua internalização, os vacúolos parasitóforos se fusionam com lisossomos formando o fagolisossomo onde ocorrem a transformação em amastigotas e se multiplicam dentro da célula-hospedeira, posteriormente levando o rompimento da célula. A lise leva a liberação dos parasitas que infectam outros macrófagos. Macrófagos infectados são ingeridos pelo inseto durante novo repasto sanguíneo, dentro do intestino do vetor os macrófagos sofrem lise liberando os parasitas que mudam da forma amastigota para promastigota não infectante. Os promastigotas passsam por um processo de fixação na parede do intestino, liberação e migração para o probóscide que é acompanhada por sua transformação em formas promastigotas metacíclicas, que é a forma infectante. O ciclo de vida completa-se quando há uma infecção de um novo flebotomíneo ao se alimentar de hospedeiros infectados ou se o vetor realizar outro repasto sanguíneo (**Figura 3**) (KAYE; SCOTT, 2011; NAGLE et al., 2014).

Figura 3- Ciclo biológico da *Leishmania spp.*



Fonte: Adaptado de NAGLE et al., 2014

Apesar da grande prevalência, não há vacinas eficazes, a maior parte dos fármacos atualmente disponíveis para o tratamento são inadequados, pois há uma alta toxicidade, custo elevado, requer um tratamento a longo prazo aplicado por via parenteral, além de não curar e eliminar os parasitas, o que está associado ao aumento de resistência dos parasitas. Estes incluem os antimoniais pentavalentes que são usados como fármacos de primeira escolha e em casos de não haver resposta ao tratamento com esses medicamentos são usados fármacos de segunda escolha como anfotericina B. Vários novos tratamentos surgiram durante os últimos 10 a15 anos, mas muitos desses não atenderam as expectativas. Entre os mesmos podem ser citados o tratamento com a paromomicina (de forma injetável e por longo tempo), a miltefosina (de custo alto, com potencial teratogênico e tratamento por longo tempo), e a anfotericina B lipossomal (também, de custo alto e com exigência de internação) (NAGLE et al., 2014, MARCHAND et al., 2015).

Na ausência de vacinas e de um tratamento eficaz, há uma preeminente necessidade de desenvolvimento de novos fármacos, menos tóxico, de baixo custo, que proporcione melhores resultados e tenha uma boa aceitabilidade pelo paciente (TIUMAN et al., 2011).

3.2. Cumarinas

A cumarina foi isolada pela primeira vez da espécie *Coumarouna odorata* (conhecida pela população por fava tonca) em 1820 por Hermann Wilhelm Vogel, membro da *Royal Academy of Science of Munich* (MURRAY, 1978).

Atualmente, já existem cerca de 1300 cumarinas identificadas de fontes naturais, como vegetais, fungos e bactérias. Elas são relatadas em cerca de 150 espécies distribuídas em cerca de 30 famílias diferentes de em plantas superiores como as famílas Asteraceae, Fabaceae, Oleaceae, Moraceae, Thymeleaceae, Apiaceae, sendo as mais ricas neste composto a Rutaceae e Umbelliferae (RADUNZ et al., 2012). Esse metabólito secundário é distribuído ao longo de todas as partes da planta, mas ocorrem em grande quantidade nas frutas, seguidos das raízes, caules e folhas. Eles estão presentes em nível elevado em alguns óleos essenciais, como o de lavanda, o de canela, na casca e nas folhas de cássia. No mirtilo, amora, chicória, chá verde entre outros alimentos existem em uma grande concentração de cumarinas (ASIF, 2015).

Nos fungos e bactérias, outras importantes cumarinas foram isoladas: a novobiocina, um antibiótico com potente inibição da DNA girase isolado de *Streptomyces* (actinobactérias de maior ocorrência no solo); as aflatoxinas, um grupo de metabólitos altamente tóxicos dos fungos (espécie *Aspergillus*), cuja ingestão pode causar danos graves a saúde humana e animal (TORTORA et al., 2011).

As cumarinas são classificadas como compostos pertencentes ao grupo das benzopironas, os quais consistem em um núcleo básico resultante da fusão dos anéis benzeno e 1,2-pirona, sendo o representante principal a cumarina, também conhecida como 1,2-benzopirona. Ele pode ser subdividido em α-benzopirona, que inclui as cumarinas e β-benzopironas, que têm os flavonóides como os principais membros (**Figura 4**) (LACY; O´KENNEDY, 2004).

Figura 4- A estrutura química das subclasses de benzopironas.

(**A**)α-benzopirona (**B**) β-benzopirona

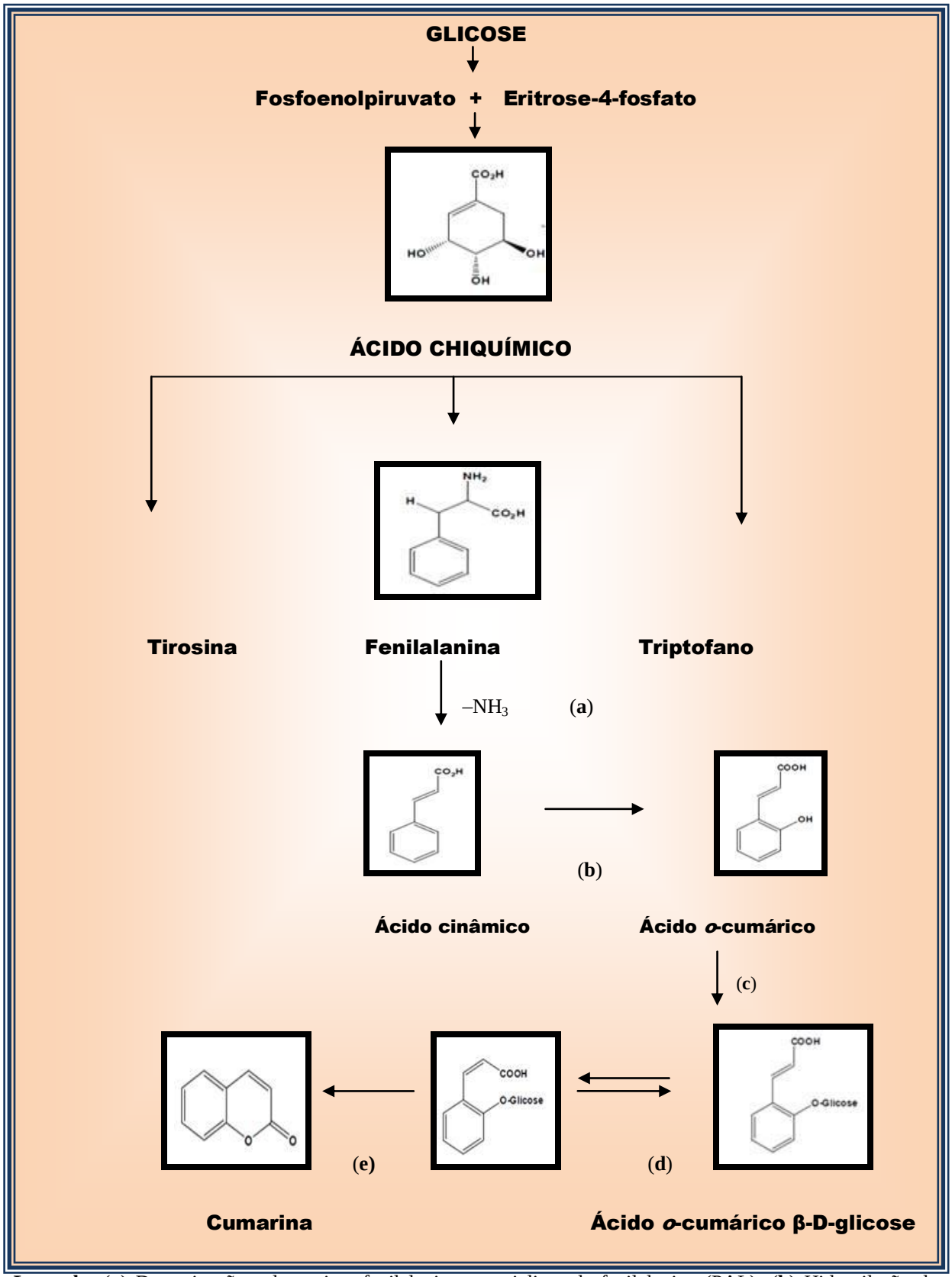
Legenda: (A) α-benzopirona, estrutura básica das cumarinas, (B) β-benzopironas, estrutura dos flavonóides. Fórmula molecular: $C_9H_6O_2$
Fonte: Adaptado de Lacy;O´Kennedy, 2004.

A biossíntese das cumarinas advém do metabolismo da glicose através de um dos dois principais intermediários, o ácido chiquímico. O ácido chiquímico é formado pela condensação de dois metabólitos da glicose: o fosfoenolpiruvato e a eritrose-4-fosfato (**Figura 5**). A via do ácido chiquímico é responsável pela formação de três aminoácidos aromáticos importantes: a fenilalanina, triptofano e tirosina que são os precursores dos metabólitos secundários das plantas como as cumarinas, flavonóides, alcalóides (CZELUSNIAK et al., 2012).

A cumarina é sintetizada através do aminoácido fenilalanina. A desaminação enzimática desse aminoácido pela ação da enzima fenilalanina amonialiase origina o ácido cinâmico. Por sua vez, o ácido cinâmico sofre hidroxilação da sua cadeia lateral, catalisada pela enzima trans-cinamato-4-hidroxilase formando o composto ácido *o*-cumárico. O derivado hidroxilado sofre *o*-glicosilação e em seguida uma isomerização cis/trans da dupla /ligação da cadeia lateral. A cumarina, resultado final desse processo, é formada pela lactonização do ácido *o*-cumárico através de ação enzimática e na presença de calor (**Figura 5**) (DEWICK, 2002; SCIO, 2004; CZELUSNIAK et al., 2012).

A biossíntese de cumarinas pode ocorrer por uma deficiência de nutrientes, alterações dos hormônios vegetais ou como resposta a algum estresse provocado por danos físicos e pragas. Um exemplo dessa resposta pode ser encontrado no girassol (*Helianthus annuus*), que acumula a cumarina escopoletina nos seus tecidos após sofrer um ataque de insetos (SIMÕES, 2010).

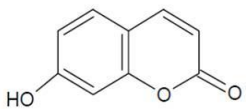
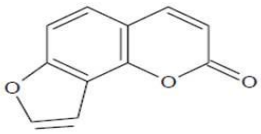
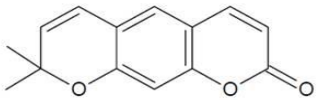
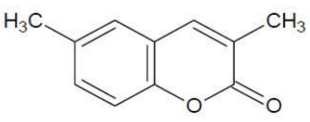
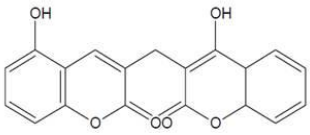
Figura 5- Rota de biossíntese da cumarina.



Legenda: (**a**) Desaminação pela enzima fenilalanina amonialiase da fenilalanina (PAL); (**b**) Hidroxilação da cadeia lateral do ácido *o*-cumárico pela enzima transcinamato-4-hidroxilase; (**c**) Glicosilação do ácido *o*-cumárico; (**d**) Isomerização cis/trans da dupla ligação; (**e**) Lactonização do produto resultante da reação.
Fonte: Adaptado de Dewick, 2002; Scio, 2004; Czelusniak et al., 2012.

A cumarina pode ser classificada em: cumarinas simples, furanocumarinas, piranocumarinas, cumarinas com substituentes no anel pirona e cumarinas miscelâneas (**Tabela 1**). As cumarinas simples são derivados que contém os radicais alquil, hidroxi e alcoxi. As furanocumarinas são compostos que possuem um anel furano condensado ao núcleo cumarínico e as mesmas podem ser classificadas em angulares e lineares de acordo com a posição do anel furano em relação aos outros anéis, além de possuir substituentes ligadas às posições dos demais carbonos do anel benzeno. As piranocumarinas são análogas da furanocumarinas. Elas contém um anel pirano (anel de seis membros consistindo de cinco átomos de carbono e um átomo de oxigênio e contém 2 duplas ligações) ligado ao núcleo cumarinico. As cumarinas com substituentes no anel pirona são as que possuem substituentes na posição 3 e 4. As cumarinas miscelâneas compreendem as biscumarinas, possuem o átomo de oxigênio ligado ao oxigênio por dupla ligação em posições invertidas da cumarina (MURRAY, 1978; DIGHE et al., 2010; VENUGOPALA et al., 2013)

Tabela 1. Diferentes tipos de cumarinas, estrutura química e suas atividades farmacológicas.

TIPOS DE CUMARINA	ESTRUTURA QUÍMICA GERAL	ATIVIDADES FARMACOLÓGICAS	REFERÊNCIAS
Cumarina simples		Anticancerígena Antibacteriana	VENUGOPALA et al., 2013
Furanocumarinas		Antiflamatória Anticonvulsivante	VENUGOPALA et al., 2013
Piranocumarinas		Antibacteriana Antiviral	VENUGOPALA et al., 2013
Cumarinas com substituentes no anel pirona		Anticancerigena	VENUGOPALA et al., 2013
Cumarinas miscelâneas		Anticoagulante	VENUGOPALA et al., 2013

3.2.1. Hidroxicumarinas

Hidroxicumarinas representam uma classe de derivados cumarínicos que têm diversas propriedades farmacológicas e bioquímicas e desempenham papéis importantes na perspectiva de compostos farmacologicamente ativos, alguns dos quais podem ser de interesse potencial farmacêutica (YASARAWAN; THIPYAPONG; RUANGPORNVISUTI, 2014).

O 4-hidroxicumarina é usado como um intermediário na síntese de diversos produtos farmacêuticos extremamente comuns, tais como a varfarina e acenocumarol, que são utilizados na prática médica como um anticoagulante (STANCHEV et al., 2009).

Umbeliferona ou 7-hidroxicumarina ocorre em plantas, frutas e raízes comestíveis, como a maçã de ouro, laranja amarga, cenoura, coentro e jardim angélica. É um sólido cristalino branco-amarelado, que tem uma ligeira solubilidade em água quente, mas alta solubilidade em etanol. Alguns de seus derivados mostram significativa atividade antioxidante e anti-inflamatório (VASCONCELOS et al., 2009).

3.2.2. Atividades biológicas das cumarinas

Diversas atividades biológicas foram atribuídas às cumarinas; entre tantas, abordaremos as seguintes atividades como antibacteriana, antifúngica, anticancerígena, antioxidante e antiparasitária.

3.2.2.1. Atividade antibacteriana

Em razão do aumento da resistência de microorganismos patogênicos a múltiplas drogas e ao uso indiscriminado de antibióticos, surge à preocupação e a procura de novas alternativas terapêuticas (LOBÔ et al., 2010), e as cumarinas têm sido relatada em diversos trabalhos com uma potencial atividade antibacteriana. Uma série de derivados de cumarinas (4-hidroxi, 7-hidroxi e 3-carboxicumarinas) demonstrou uma melhor atividade contra bactérias Gram positivas ao invés de Gram negativas, enquanto alguns foram mais eficazes, especificamente, para o *Bacillus subtilis* e *Staphylococcus aureus* (LIN et al., 2012).

Derivados cumarínicos formados a partir da molécula 4-hidroxicumarina mostraram atividade contra bactérias patogênicas importantes. Todos os compostos sintetizados a partir

dessa molécula foram ativos contra o *Bacillus atrophaeus* e *Bacillus subtillus*, alguns deles apresentaram atividade moderada contra *Pseudomonas aeruginosa* e *Escherichia coli* (REHMAN et al., 2013).

3.2.2.2. Atividade antifúngica

Extratos brutos e frações de cinco espécies do gênero *Polygala* (*P. campestris, P. cyparissias, P. paniculata, P. pulchella, P. sabulosa*- planta encontrada em Santa Catarina, Brasil) foram investigados quanto a sua atividade *in vitro* contra várias espécies de fungos oportunistas. O extrato hexânico (*P. paniculata*) e a fração de acetato de etila (*P. sabulosa*) mostraram uma melhor concentração inibitória mínima (CIM) de 60 μg/mL para *Candida tropicalis* e 30 μg/mL para *Cryptococcus gattii*. Devido a isso, foram isoladas as possíveis substâncias que caracterizavam essa atividade, e dentre elas se destacaram alguns compostos cumarínicos que exibiram isoladamente uma excelente CIM. A 6-metoxi-7-preniloxicumarina, isolada da *P. sabulosa*, apresentou uma CIM de 250 μg/mL para o *Sporothrix schenckii* e *C. gattii* e o aurapteno, isolada da *P. paniculata*, um valor de CIM de 250 μg/mL para o *C. gattii* (JOHANN et al., 2011).

A cumarina, isolada da fração acetona de *Ageratum conyzoides* L., atuou como fungicida contra a *Candida albicans* com o CIM de 125 μg/mL. Esse efeito foi também observado através de estudo com microscopias eletrônicas de transmissão e varredura, em que a maioria das células apresentaram poros e espessamento da parede celular, além da redução da densidade citoplasmática, vacúolos mais largos e necrose do conteúdo citoplasmático (WIDODO et al., 2012).

Em outro estudo, dois novos compostos cumarínicos obtiveram êxito com uma atividade antifúngica significativa quando comparada ao fluconazol. Essas duas substâncias foram sintetizadas por Al-Amiery e colaboradores (2012) e testadas contra as espécies de *Candida albicans* e de *Aspergillus niger*. A correlação entre a estrutura química e a atividade biológica revelaram que a característica essencial da sua ação farmacológica é a presença de derivados amino-substituídos.

A cumarina mammeisin foi isolada da *Kielmeyera elata* e testada a sua ação antifúngica. A concentração inibitória mínima exibida por essa cumarina foi de 512 μg/mL

para todas as quatro espécies de *Candida* sp testadas (*C. albicans, C. tropicalis, C. parapsilosis* e *C. krusei*). Essa atividade foi equivalente quando comparada com o controle positivo (cetaconazol), mas com melhores resultados com relação ao fluconazol (MARCONDES et al., 2015).

3.2.2.3. Atividade antioxidante

Espécies reativas de oxigênio (EROs) inclui as espécies de radicais livres e outras que, embora não possuam elétrons desemparelhados, são muito reativas em decorrência de sua instabilidade. Essas moléculas são produzidas continuamente pelas células do corpo humano e concentrações fisiológicas delas tem funções biológicas definidas, atuando como mensageiros de sinalização. Entretanto, o aumento de sua concentração deve ser evitado pelo organismo, considerando que sua reatividade traz consequências que causa a oxidação de biomoléculas tais como proteínas, lipídeos, carboidratos, DNA e o rompimento da homeostase celular (WU et al., 2007; ZHANG et al., 2011).

Para se defender dessa toxicidade, o organismo apresenta uma proteção antioxidante que é feita por moléculas que protegem alvos biológicos da oxidação, por apresentarem uma das três propriedades: supressão da formação de espécies reativas de oxigênio (EROs) através da quelação de metais ou inibição de enzimas geradoras de radicais livres; eliminação ou desativação de EROs, formando um produto estável e participando do processo de reparo de dano as biomoléculas atingidas (RIBEIRO et al., 2005).

Há uma variedade de moléculas com potencial por apresentar uma destas características, incluindo algumas do próprio organismo (enzimas antioxidantes como a glutaniona peroxidase) e outras oxogénas, sintéticas e naturais. Verificou-se que as cumarinas são uma classe de substâncias de diversas estruturas químicas e exibem essa importante característica de proteção antioxidante (RIBEIRO et al., 2005; SANTOS et al., 2014) e foi mostrado por Kancheva e colaboradores (2010) através de seus estudos a capacidade antioxidante *in vitro* através do método do DPPH (2,2-difenil-1-picrilidrazil) de cinco novas 4-hidroxi-bis-cumarinas, sendo que dois compostos apresentaram uma atividade bastante elevada com relação às outras substâncias.

3.2.2.4. Atividade anticancerígena

O câncer é uma das principais riscos de saúde e das causas de mortes proeminente no mundo. A maioria dela ocorre por desregulação das enzimas essenciais e outras proteínas que controlam a divisão celular, o que leva a um descontrole no crescimento e alteração na proliferação das células. Apesar de muitos esforços para o tratamento adequado e o diagnóstico, alguns pacientes não respondem a terapia ou ocorre à reincidência do câncer (EMAMI; DADASHPOUR, 2015).

Um certo número de quimioterápicos estão atualmente em prática clínica e ainda é uma abordagem básica e de grande relevância para o tratamento, no entanto existem obstáculos encontrados como resistência aos múltiplos agentes anticancerígenos, toxicidade induzidas das drogas, alta incidência de efeitos colaterais. Deste modo, a descoberta de novos agentes com atividade promissora e elevado índice terapêutico para essa doença é de uma necessidade urgente. Devido a sua potencial aplicação como anticancerígenos, grandes esforços têm sido realizados para o estudo desses derivados cumarínicos, além da sua concepção e síntese (VIJAYARAGHAVALU et al., 2012).

A cumarina é um dos compostos importantes encontrada na canela através de técnicas espectroscópicas e foi utilizada por Ahmad e colaboradores (2014) para avaliar a atividade anticancerígena sobre a linhagem celular A2780 de carcinoma epitelial do ovário humano. A cumarina extraída inibiu a proliferação de 50% das células *in vitro* na concentração de 0,64 mg/mL.

As atividades antiproliferativas *in vitro* e *in vivo* da esculetina foram avaliadas contra o carcinoma hepatocelular e foi revelada uma inibição bastante significativa para essa cumarina testada (WANG et al., 2015).

Kim e colaboradores (2015) observaram que a esculetina (6,7-dihidroxicumarina) possuem um potente efeito citotóxico sobre a linhagem HT-20 (adenocarcinoma de cólon retal humano) com um efeito do tipo dose e tempo-dependente. O tratamento com 55 µg/mL reduziu a viabilidade celular em 50%. Nessa dose, houve a indução da apoptose demonstrada através da condensação da cromatina nos núcleos, fragmentação do DNA, além da indução de perda do potencial de membrana mitocondrial.

3.2.2.5. Atividade antiparasitária

Cercárias de *Schistosoma mansoni* são capazes de produzir eicosanóides, substâncias necessárias para a sua penetração na pele do hospedeiro e trabalhos realizados por Salafsky e Fusco (1985) demonstraram que a esculetina foi capaz de diminuir essa formação de eicosanóides, mesmo quando as cercárias foram estimuladas por fatores externos como o linoleato.

Toddalia asiatica (L) Lam (Rutaceae) é uma planta usada por várias comunidades no tratamento da malária e outras doenças no Quênia. Todas as partes da planta têm valor medicinal, mas se acredita que as raízes têm um maior potencial antimalárico. Devido a esse fator, Oketch-Rabah e colaboradores (2000) fizeram um estudo com os extratos (metanol, diclorometano e acetato de etila) da raiz dessa planta. O extrato metanólico mostrou uma atividade antiplasmódica elevada e a partir desse extrato foi isolada o 5,7-dimetoxi-8-(3-hidroxi-3-metil-1-buteno)-cumarina que apresentou uma atividade bastante elevada contra o *Plasmodium falciparum*.

A cumarina 4-(1-metilpropil)-5,7-di-hidroxi-8-(4-hidroxi-3-metilbutiril)-6-(3-metilbut-2-enil) cromen-2-ona, isolada da espécie *Kielmeyera albopunctata* presente no Cerrado brasileiro, produziu uma taxa de mortalidade de 80% da forma tripamastigota do *Trypanosma cruzi*, em um período de 24 horas com uma concentração de 125 μg/mL (SCIO et al., 2003).

Pizzolatti e colaboradores (2008) realizaram um estudo com quatro frações (etanol, hexano, acetato de etila e diclorometano) de *Polygala sabulosa,* no qual o diclorometano apresentou um resultado semelhante aos controles positivos (benzonidazol e violeta de genciana) contra as formas epimastigotas e tripomastigotas do *Trypanosoma cruzi* (IC_{50}: 10,4 e 147,6 μg/mL, respectivamente). Devido a esse bom resultado, isolaram-se várias substâncias presentes na fração de diclorometano, entre elas, a 6-metoxi-7-preniloxicumarina. A 6-metoxi-7-preniloxicumarina mostrou interessante atividade tripanocida contra as formas epimastigotas (IC_{50} = 10,5 μg/mL) e tripamastigotas (IC_{50} = 88,2 μg/mL).

Cumarinas isoladas da *Helietta apicultata* Benth. (Rutaceae), a escopoletina e a escoparona, demonstraram uma moderada atividade contra a forma promastigota da *Leishmania amazonensis*, *Leishmania infantum* e *Leishmania braziliensis* com o valor da IC_{50} maior que 50 μg/mL (FERREIRA et al., 2010).

A escoparona (6,7-dimetoxi-cumarina), isolada da *Platymiscuim floribundum*, apresentou atividade contra espécies do Gênero *Leishmania*. As espécies testadas demonstraram ter diferentes susceptibilidades ao composto-teste. A *Leishmania mexicana* foi mais sensível do que a *Leishmania major* e a *Leishmania donovani* (VILA-NOVA et al., 2013).

Bashir e colaboradores (2014) observaram a atividade antiprotozoária de três derivados de cumarinas (conferol, conferona e umbeliferona), isolados da *Ferula narthex* Boiss, quando testados contra a *Leishmania major*. O conferol se mostrou mais potente com a obtenção da IC_{50} na concentração de 11,51 $\pm$ 0,09 µg/mL.

4. MATERIAL E MÉTODOS

4.1. Local de realização dos experimentos

Os experimentos foram realizados prioritariamente no Laboratório de Cultura de Tecidos do Departamento de Histologia e Embriologia da Universidade Federal de Pernambuco (LCT-DHE). Colaborações foram realizadas com pesquisadores do Setor de Microscopia Eletrônica do LIKA-UFPE.

4.2. Compostos-testes

Os compostos (1,2-benzopirona, 3-hidroxicumarina e 4-hidroxicumarina) foram cedidos pelo Professor Dr. José Maria Barbosa Filho do Laboratório de Tecnologia Farmacêutica da Universidade Federal da Paraíba (LTF-UFPB).

4.3. Avaliação da atividade leishmanicida *in vitro*

4.3.1. Cultivo das formas promastigotas da *L.(L.) amazonensis*

Os parasitas foram cedidos gentilmente pelo Dr. Osvaldo Pompílio de Melo Neto do Departamento de Microbiologia do Centro de Pesquisa Aggeu Magalhães (UFPE). As formas promastigotas da *Leishmania (Leishmania) amazonensis* (MHOM/77/BR/LTB0016) foram cultivadas em meio LIT (*Liver Infusion Tryptose* – HiMedia, Laboratories Pvt. Ltda., Mumbai, India) suplementado com 10% de Soro Fetal Bovino inativado (Invitrogen, Califórnia, USA), 0,2% de hemina e 0,1% de antibióticos (100UI/mL de penicilina e 100 µg/mL de estreptomicina, Gibco BRL, Life Technologies, Paisly, UK), a 26°C em estufa B.O.D.

4.3.2. Atividade antipromastigota pelo método do MTT

A obtenção da IC_{50} (concentração da substância que inibe 50% do crescimento dos parasitas em relação ao controle) foi realizada com o método colorimétrico do MTT

(MOSMANN, 1983). O brometo de 3-metil [4,5-dimetiltiazol-2-il]-2,5 difeniltetrazólio, conhecido como MTT (um sal de coloração amarela e solúvel em água), possibilita a investigação da atividade metabólica das células com base na redução desse sal por desidrogenases, resultando na produção de cristais de formazan (de cor arroxeada e insolúvel em água) que é proporcional ao número de células metabolicamente ativas.

Os parasitas em fase exponencial de crescimento (três dias) foram distribuídos numa concentração de 1 x 10^5 parasitas/mL em placas de 96 poços de fundo chato junto com os compostos em estudo. Para os ensaios, as substâncias foram dissolvidas em dimetilsulfóxido (DMSO) (Vetec Química Fina, Sigma-Aldrich, St Louis, MO, USA) e em seguida diluídos em meio LIT nas concentrações de 1,56 a 400 µg/mL (3-hidroxicumarina e 4-hidroxicumarina). A Anfotericina B (Sigma-Aldrich, St Louis, MO, USA) foi utilizada como controle positivo e como controle negativo foi utilizado meio de cultura e o solvente DMSO (0,5%). A placa contendo os parasitas e os extratos foram incubadas a 26°C em estufa incubadora B.O.D (Caltech Indústria e Comércio LTDA, Franca, São Paulo, Brasil) durante 72 horas.

Após 72 horas de incubação, foi adicionado a cada poço 20µL de MTT (5mg/mL) e as placas foram incubadas por três horas em estufa BOD a 26°C. Após esse período, o meio de cultura e o excesso do MTT foram aspirados e adicionados 100 µl de DMSO por poço durante 30 minutos para dissolver os cristais de formazan. As placas foram lidas em uma leitora de microplacas (SkanIt Software 2.4.5 RE for Varioskan Flash, Thermo Scientific, Massachusetts, USA) em 595 nm. Foram realizados três experimentos em triplicatas.

4.4. Avaliação da atividade citotóxica *in vitro*

4.4.1. Cultura de células

As células Vero (células epiteliais do rim do macaco verde africano, *Cercopithecus aetiops* adulto normal), as células HeLa (carcinoma cervical humano) e as células J774 (macrófagos murinos) foram cultivadas em meio DMEM suplementado com 10% de Soro Fetal Bovino inativado e 0,1% de antibióticos em estufa de CO_2 a 5%, 95% de umidade a 37ºC. A citotoxicidade foi determinada pelo método colorimétrico de MTT. As células foram adicionadas nas concentrações de 1 x 10^5 (célula Vero) e 2 x 10^5 células/mL (célula HeLa e J774) em placas de 96 poços onde foram adicionadas as concentrações de 0,78 a 400 µg/mL

da substância-teste (3-hidroxicumarina). Como controles negativos foram usados o meio DMEM e o DMSO a 0,5%. A leitura da absorbância em espectrofotômetro no comprimento de 595 nm. Foram realizados três experimentos em triplicatas.

4.5. Análise morfológica das formas promastigotas de *L. (L.) amazonensis*

4.5.1. Microscopia invertida com contraste de fase

A morfologia das células foi avaliada através de sistema vídeo-microscopia com o auxílio do microscópio invertido com contraste de fase LEICA (Leica microsystems, Wetzlar, Alemanha) acoplado a uma câmera (MOTICAM BA 2000, Campinas, Brasil) e foram realizados registros fotográficos (Motic Images Plus 2.0 software) de cada concentração testada bem como do controle (DMSO a 0,5%).

4.5.2. Microscopia Eletrônica de Varredura (MEV)

As amostras foram fixadas em solução de glutaraldeído a 2,5%, paraformaldeído a 4% em tampão cacodilato de sódio a 0,1M, pH 7,4; lavadas 3 vezes a cada 10 minutos no mesmo tampão e pós-fixadas em Tetróxido de Ósmio (OsO4) a 1% em tampão cacodilato de sódio a 0,1M, pH 7,2 por 1 hora. Após esta etapa, o material foi lavado três vezes a cada 10 minutos no mesmo tampão, desidratado em séries crescentes de etanol (30, 50, 70, 90 e 100%) e posteriormente as amostras seguiram para o ponto crítico. Após a montagem e metalização, o material foi observado no MEV (JEOL JSM T-200, Tokyo, Japan).

4.6. Avaliação da atividade antioxidante

A capacidade antioxidante foi avaliada através do método do sequestro de radicais livres do 2,2-difenil-1-picrilhidrazil (DPPH, Sigma-Aldrich) como previamente descritas por Blois (1953). Uma alíquota de 250 µl de solução de DPPH (1 mM) foi misturada com 40 µL de diferentes concentrações de cumarina e seus derivados (31,2 a 1000 µg / ml). Após trinta minutos, a absorbância foi medida a 517 nm. O Trolox foi usado como composto referência e para o branco foram adicionados 40 µL do solvente. A atividade antioxidante foi calculada em porcentagem mediante a seguinte fórmula (JANU et al., 2013):

Atividade antioxidante [DPPH] (%) = (Ac – As) / Ac × 100

Onde Ac é a absorbância do controle, As é a absorbância das amostras.

4.7. Avaliação da atividade hemolítica

A atividade hemolítica foi determinada pelo método descrito por Figueirôa e colaboradores (2013). Sangue (5-10 mL) foi obtido a partir voluntários saudáveis, não-fumantes, por punção venosa e colocado em tubos heparinizados, após consentimento informado por escrito. Eritrócitos humanos foram isolados por centrifugação (1000 x *g*, 10 min a 4 ° C). Após a remoção do plasma, os eritrócitos foram lavados três vezes com solução salina tamponada com fosfato (PBS; pH 7,4). Uma alíquota de 1,1 mL de suspensão de eritrócitos foi misturada (1%) a 0,4 mL de cumarina e seus derivados (31,2 a 1000 µg/ml). Os controles positivo e negativo são, respectivamente, o Triton X-100 e o DMSO. Após 60 min de incubação, as células foram centrifugadas e a absorbância do sobrenadante foi registada a 540 nm. O valor médio foi calculado a partir dos ensaios em triplicata. A atividade hemolítica foi expressa em relação à ação do Triton X-100 e calculada pela seguinte fórmula:

Atividade Hemolítica (%) = [(As - Ab) × 100] / (Ac - Ab)

Onde As é a absorbância do controle negativo (branco, sem extrato), Ab é a absorbância das amostras e Ac é a absorbância do controle positivo.

4.8. Análise estatística

4.8.1. Análise estatística referente ao Manuscrito 1

Os valores das concentrações efetivas para a atividade citotóxica foram calculados no programa Origin 8.1 e a significância estatística das diferenças entre os grupos foi avaliada pela ANOVA, seguido do teste de *Tukey* com valor de $p<0,05$.

4.8.2. Análise estatística referente ao Manuscrito 2

Os valores das concentrações estão representados como média ± desvio padrão de três experimentos independentes. As diferenças estatísticas entre os grupos foram determinadas pelo Teste *t* de Student. O método de ANOVA foi usado para encontrar a significância da diferença entre os valores. Um valor de $p<0,05$ é considerado estaticamente significante e foi calculado no programa Origin 8.1. A concentração de inibição de 50% do crescimento dos parasitas (IC_{50}) e das células foi calculada por interpolação linear.

5. RESULTADOS E DISCUSSÃO

O resultado do presente estudo está descrito em formato de artigos submetidos a revistas científicas expostas abaixo:

MANUSCRITO 1

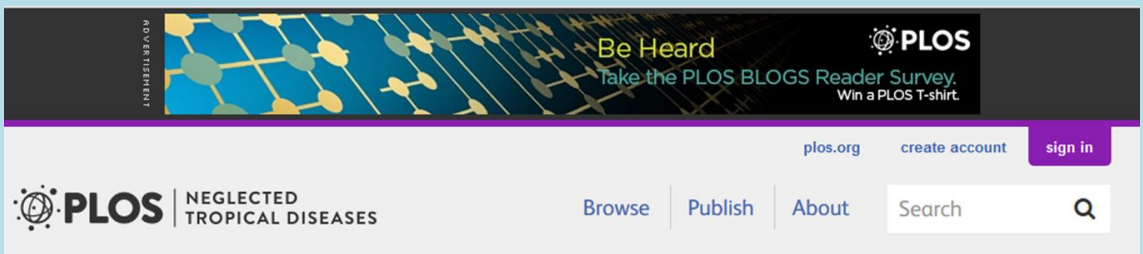
Título: *In vitro* cytotoxicity of 3-hydroxycoumarin on Vero and HeLa cell lines

Submetido a revista científica Evidence-Based Complementary and Alternative Medicine (eCAM), de acordo com o Qualis Capes 2014 é definida como B2 no comitê de farmácia.

MANUSCRITO 2

Título: Natural and Synthetic Coumarins derivates with Potential Activity against *Leishmania (L.) amazonensis* promastigotes

A ser submetido a revista científica PLOS Neglected Tropical Diseases, de acordo com o Qualis Capes 2014 é definida como A1 no comitê de farmácia.



Manuscrito 1

In vitro cytotoxicity of 3-hydroxycoumarin on Vero and HeLa cell lines

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Abstract

Natural products have been considered good tools for prospecting of new active drugs or models for new therapeutic drugs. Coumarins are a group of natural phenolic compounds that shows several pharmacological activities and the 3-hydroxycoumarin represents as target valuable molecules against several diseases. The aim of present work was to investigate the *in vitro* cytotoxic activity of 3-hydroxycoumarin on Vero and HeLa cells and to evaluate the morphological aspects of these cell lines. Cytotoxicity was measured using MTT colorimetric assay and the morphological features of these cells were evaluated by phase-contrast microscopy. The results of this first study have demonstrated to cytotoxic activity of 3-hydroxycoumarin on Vero and HeLa cell lines. MTT assay provides information regarding the cytotoxic activity and the proliferation of Vero and HeLa cells was significantly (p<0.05) inhibited from 100 µg/mL of 3-hydroxycoumarin, revealing morphological changes in high concentrations (100 to 400 µg/mL). Based on the results, more studies are required to elucidate the mechanism 3-hydroxycoumarin action in a comprehensive manner.

Keywords: 3-hydroxycoumarin; Vero cells; HeLa cells; cytotoxic activity; morphological analysis.

1. Introduction

The use of plants based in folk medicine have been a major target of the pharmaceutical industry which has been trying to find new prototypes useful for the drugs directed to the treatment of various diseases. This has led to a resurgence of interest in secondary metabolites produced as phenolic compounds, among which highlight the coumarin [1]. These purified substances exhibit potent and relevant biological activities, in addition to its low mammalian toxicity. This set of benefits keeps the coumarins as research target on current research and promotes pharmaceutical interest worldwide [2].

Coumarins (2H-1-benzopyran-2-one) owe their class name to "Coumarou", the vernacular name of the tonka bean, *Dipteryx odorata* (Aubl.) Willd. (Fabaceae), from which coumarin itself was isolated in 1820 [3]. Coumarins are distributed in nature and are a class of natural phenolic substances found in plants [4], bacteria [2] and fungi [5], widely used as additives in food, perfumes, cosmetics, pharmaceuticals [6]. Nearby, 1.300 coumarins were identified from natural resources and reported in about 150 species distributed in 30 different families in higher plants, richest sources being Rutaceae and Umbelliferone. This secondary metabolite is distributed over all parts of the plant, but occurs in large quantities in fruits, followed by the roots, stalksand leaves. [7].

Coumarin and its derivatives have a large number of properties and applications that justify the interest in these compounds. Diverse biological properties are attributed, such as anticoagulant [8], antifungal [9], anticonvulsant [10], antitubercular [11], antiadipogenic [12], antihypertensive [13], antihyperglycemic [14], antioxidant [15], anti-inflammatory [16], antibacterial [17], anticancer [18], antiviral [19], photoprotective [20]. However, some pharmacological properties of certain coumarins and its derivatives have not been investigated, such as the cytotoxic activity.

Hydroxycoumarins represent a class of coumarin derivatives that have diverse pharmacological and biochemical properties and play important roles in the prospect of pharmacologically active compounds, some of which may be of potential pharmaceutical interest [21]. The 4-hydroxycoumarin is used as an intermediate in the synthesis of various extremely common pharmaceuticals, such as warfarin and acenocoumarol, which are used in medical practice as an anticoagulant. Some derivatives of 7-hydroxycoumarin (umbelliferone) show significant antioxidant activity and anti-inflammatory [22, 23].

Although some studies have showed several biological activities of natural and synthetic coumarin derivatives, addictionally the activity of certain hydroxycoumarins such as 3-hydroxycoumarin has not been investigated. In this context, the present study aimed to evaluate the *in vitro* cytotoxic activity of 3-hydroxycoumarin on Vero and HeLa cells and to evaluated the morphological aspects of these cell lines.

2. Materials and Methods

2.1. Chemicals. 3-hydroxycoumarin was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO) and stored at 5°C. MTT powder [3-4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide], cell culture medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin-EDTA, penicillin-streptomycin mixture and L-glutamine were from Gibco BRL (Life Technologies, Paisly, UK).

2.2. Cell culture. HeLa (Human cervical carcinoma) and Vero cells (*Cercopithecus aethiops* Green monkey kidney epithelial cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Gibco BRL, Life Technologies, Paisly, UK). The culture was maintained at 37 ºC in an atmosphere of 5% CO_2 and 95% of relative humidity.

*2.3. Cell viability assay.*The cytotoxic activity *in vitro* was evaluated using the MTT assay. Briefly, Vero cell ($1x10^5$ cells/mL) and HeLa cell ($2x10^5$ cells/mL) were seeded in 96-wells plates and incubated for 24 h. 3-hydroxycoumarin was dissolved in dimethyl sulfoxide and added in different concentrations (0.78- 400 µg/mL). As controls, we used DMSO or DMEM. The compounds dissolved were applied to culture wells in triplicate and incubated for 72 h. The formation of formazan was measured by adding 20 µL de MTT (5mg/mL) to each well and the cells were incubated at 37°C in the dark for 3h. After this time, all supernatant was discarded and subsequently the formazan crystals were dissolved in DMSO (100 µL). The optical density of each well was measured at 595 nm using an ELISA reader (SkanIt Software 2.4.5 RE for Varioskan Flash, Thermo Scientific, Massachusetts, USA).

2.4. Morphological analysis. Vero and HeLa cell lines were cultured in 96-well plates for 72 h, in the presence or absence of different concentrations of 3-hydroxycoumarin. After treatment, the morphological features of these cells were evaluated by phase-contrast microscopy using an inverted microscope LEICA (Leica microsystems, Wetzlar, Germany), equipped with digital camera (MOTICAM BA 2.000, Campinas, Brazil) and the digital photographs were taken using the Motic Images Plus 2.0 software.

2.5. Statistical Analysis. Data were analyzed using Origin 8.2 program. All results obtained in this study are presented as mean ± standard error of the mean (SEM) of experiments performed in triplicate. The values of groups were compared using the one way analysis of variance (ANOVA) followed by *Tukey*'s post-test. **p*<0,05 was considered to statistically significant.

3. Results and Discussion

3.1. C*ytotoxic activity of 3-hydroxycoumarin on Vero cell line.*

The cytotoxicity of 3-hydroxycoumarin in Vero cells was determined by the colorimetric method MTT, where the cells were distributed in 96-well microtiter plates and their proliferation was evaluated with 72h [24]. The percentage of Vero cells viability was established according to the concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) of 3-hydroxycoumarin (figure 1). Our results are statistically significant (p<0.05) when compared to control (DMSO).

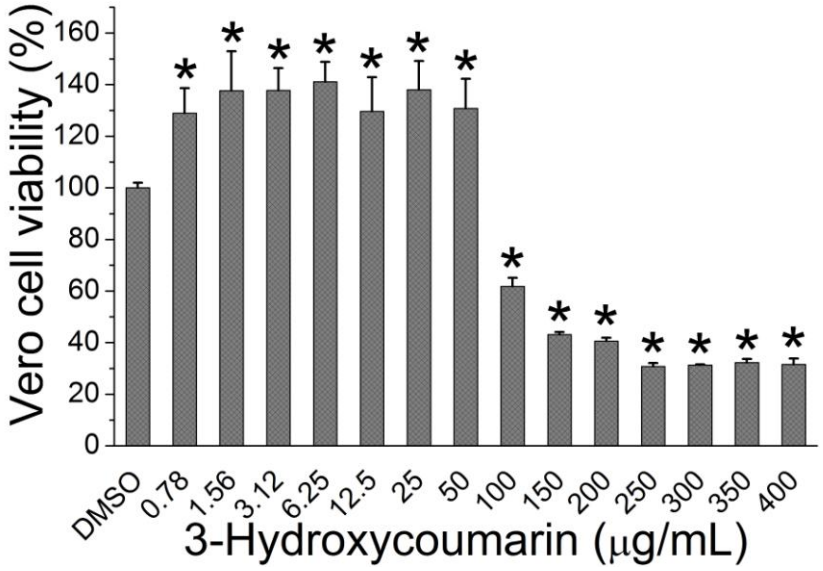

Figure 1. Effect of 3-hydroxycoumarin on *in vitro* viability (%) of Vero cell lines. The data presents means ± standard error of the mean of at least of at three independent experiments performed in triplicate. *p<0.05 compared to control group (one-way ANOVA followed by Tukey's post-test).

The lowest concentrations of 3-hydroxycoumarin (0.78 to 50 µg/mL) exhibited viability higher than those of the control (DMSO). The results obtained in this work, suggested that the 3-hydroxycoumarin could promote or inhibit the viability of normal cells in a concentration-dependent manner. However, the higher concentrations of 3-hydroxycoumarin (100 to 400 µg/mL) led to a significant decrease in the Vero cell viability.

Coumarins have shown anti-oxidant or pro-oxidant properties depending on their intracellular concentration and its derivatives may present an antioxidant activity at low concentrations and pro-oxidant effect at higher concentrations which induces an intracellular overproduction of Reactive Oxygen Species (ROS) leading to cell death [25]. In our experiments, the effect of the higher concentrations of 3-hydroxycoumarin (100 to 400 µg/mL) could be associated with an decrease in antioxidant defense capacity, which probably may exceed the production of ROS leading to cell death.

Recent studies reported that a new series of chalcone-coumarin derivatives showed high cytotoxicity to cancer cells (HuCCA-1, Hep-G2, A549) and is non-toxic to normal cells

(Vero cells). Molecular docking studies have revealed that specific action in cancer cells might possibly be due to a dual inhibition of both binding sites (colchicine and GTP) on the α and β-tubulin [26].

3.2. C*ytotoxic activity of 3-hydroxycoumarin on HeLa cell line*

The results of cytotoxic assay of 3-hydroxycoumarin on cells of the human cervical carcinoma (HeLa cells) exhibited effects statistically significant (p<0.05) with concentrations from 100 µg/mL and our study represents the first report demonstrating to cytotoxic activity of 3-hydroxycoumarin on HeLa cell line (Figure 2).

The coumarins and their derivatives may exert anticancer activity through several mechanisms: inhibition of telomerase enzyme, down regulation of oncogene expression [27] and among the hydroxycoumarins, the 7,8-hydroxycoumarin may demonstrate this action by generating oxidative stress due to production of free radical species in cancer cells, which leads to a pro-apoptotic effect in U-937 and HL-60 cells [28].

Coumarin and 7-hydroxycoumarin at 10-160 µg/mL induced a dose-dependent growth-inhibition in lung carcinoma cell lines and at high concentrations (> 100 µg/mL) morphological changes were observed [29]. It has been too reported in literature that esculetin (6,7-di-hydroxycoumarin) inhibits cell growth and cell cycle progression by inducing arrest in G1 phase in leukaemia HL-60, and CCRF-HSB-2 cell lines [30, 31]. This observation can be related to the presence of catecholic functions as structural requeriment for marked cytotoxic effects.

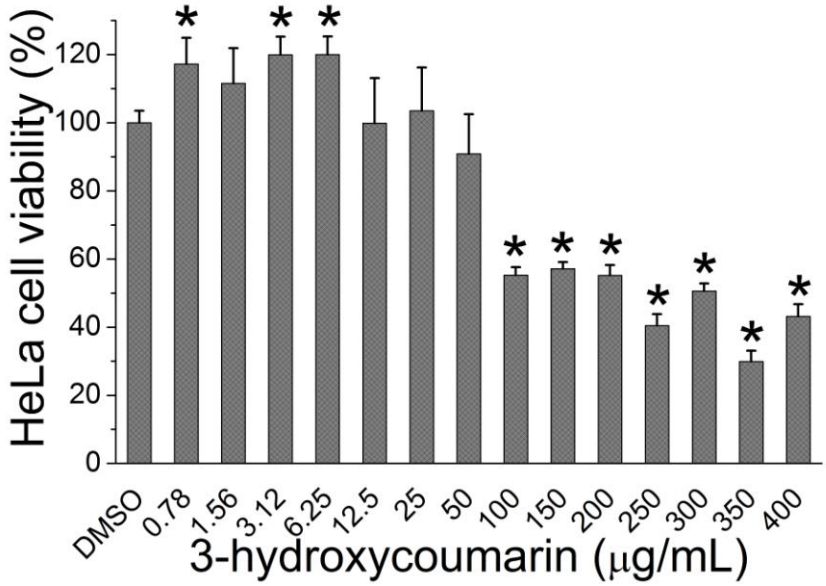

Figure 2. Effect of 3-hydroxycoumarin on *in vitro* viability of human cervical carcinoma cells (HeLa cells). The data are presented as percentage of increase (% viability) for each concentration of 3-hydroxycoumarin used in the control (DMSO). *, represent (p<0.05) significant *versus* control, an analysis of variance (ANOVA) test followed by Tukey's.

3.3. Morphological aspects of Vero cells under the action of 3-hydroxycoumarin

The effect of different concentrations of 3-hydroxycoumarin on Vero cell lines is illustrated in Figure 3. Cells were cultivated as adherent monolayer at lower concentrations of 3-hydroxycoumarin (0.78 to 50 µg/mL), identical like those observed in control. These results were consistent with the cytotoxic findings of the study in question. Possibly, the protective effect may be related to antioxidant activity of coumarin derivatives.

Phenolic compounds are bioactive substances that have one or more aromatic rings in their structure, bearing one or more hydroxyl groups. This family of compounds acts as antioxidants and thereby protect from degenerative diseases in which reactive oxygen species (ROS) are involved [32]. In fact, overproduction of free radicals can cause oxidative damage to biomolecules, (lipids, proteins, DNA), eventually leading to many chronic diseases [33].

The properties of phenolic compounds are related to their chemical structure, which confers stability to the secondary free radical formed from the antioxidant reaction product with a free radical [34]. In the context, the hydroxycoumarins are phenolic compounds which act as capacity metal chelators and free radical scavengers [35-37].

Morphological changes included retraction of cytoplasmic extensions that was observed from the concentration of 100 µg/mL. Moreover, especially at the highest concentrations (250 to 400 µg/mL), dramatic changes in Vero cell morphological features was observed revealing a decrease in cell density, as well cell rounding and shrinking. The cells demonstrated failure to reestablish intercellular associations and the growth pattern as adherent monolayer. It has been reported that deleterious effects of ROS on human cells may end in oxidative injury leading to programmed cell death [38].

Very few systematic studies have been reported on structure-antioxidant activity correlations in coumarins, but their activity is probably due to their structural analogy with flavonoids and benzophenones [39]. Therefore, the coumarins possess a great structural diversity, since the replacements can occur at any of the six available sites of their basic molecular moiety (1, 2-benzopyrone) [40].

A variety of synthesized coumarin derivatives have been experimentally shown to biological and pharmacological activities including anti-inflammatory, anticoagulant, anticancer and Alzheimer’s disease inhibition [41].

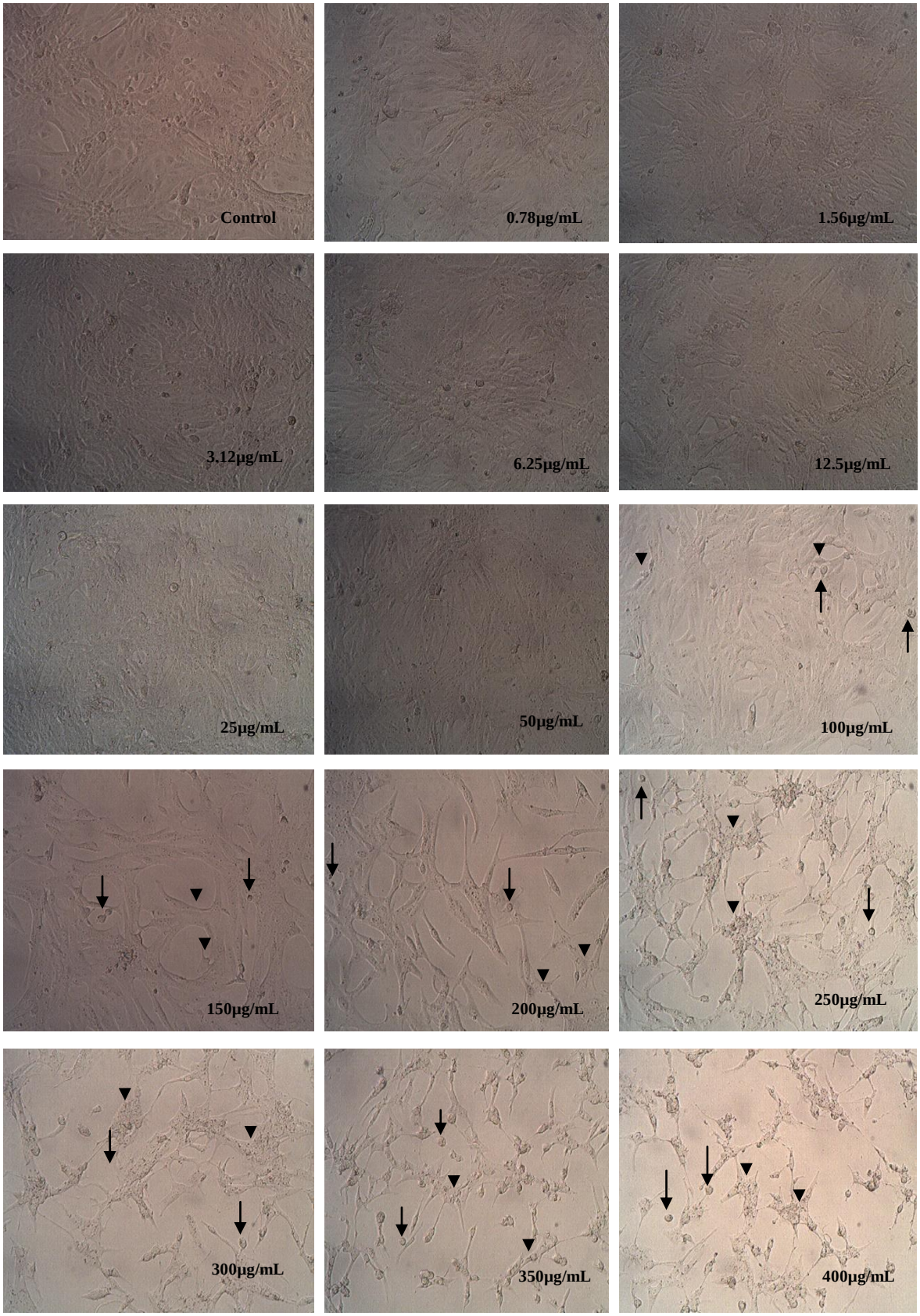

Figure 3. Phase-contrast photomicrographs of control (DMSO) and 3-hydroxycoumarin-treated Vero cell lines. Different concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) was used. Note cells cultivated as adherent monolayer at 0.78 to 50 µg/mL. Morphological alterations were observed revealing a decrease in cell density, as well cell rounding (arrow) and shrinking (arrowhead) at the highest concentrations (100 to 400 µg/mL). Magnitude of all photos: 100X.

3.4. Morphological aspects of HeLa cells by the action of 3-hydroxycoumarin

The morphological aspects of HeLa cells under effect of 3-hydroxycoumarin at different concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) were evaluated with a phase-contrast microscope. The results showed that HeLa cells were cultured as adherent monolayers at low-dose effects of 3-hydroxycoumarin (0.78 to 50 µg/mL). Morphological alterations were observed at the highest concentrations (100 to 400 µg/mL) revealing a decreased overall cell density as well as cell clusters with condensed chromatin, nuclear segmentation, shrinking and cellular debris (figure 4).

Changes in the morphology of HeLa and MCF-10A cells were also induced by betaine treatment (at 0-100 µg/mL or 24-96h) and at 100 µg/mL the cells showed nucleus morphological changes associated with apoptosis such as nuclear condensation and fragmentation and apoptotic bodies [42]. Betaine and coumarin are components derived from *Lycium chinense* an *Angelicae decursiva* (respectively) and these plants have been used for treatment of respiratory diseases in oriental medicine due to have various bioactivities effects [43]

It was interesting, too, to see our work in agreement to others researches, which refers to 7-hydroxycoumarin and 6,7-dihydroxycoumarin (esculetin) like two coumarin derivatives that have been reported to exhibit antitumor activity, but the action mechanism underlying this activity remains unknown [44].

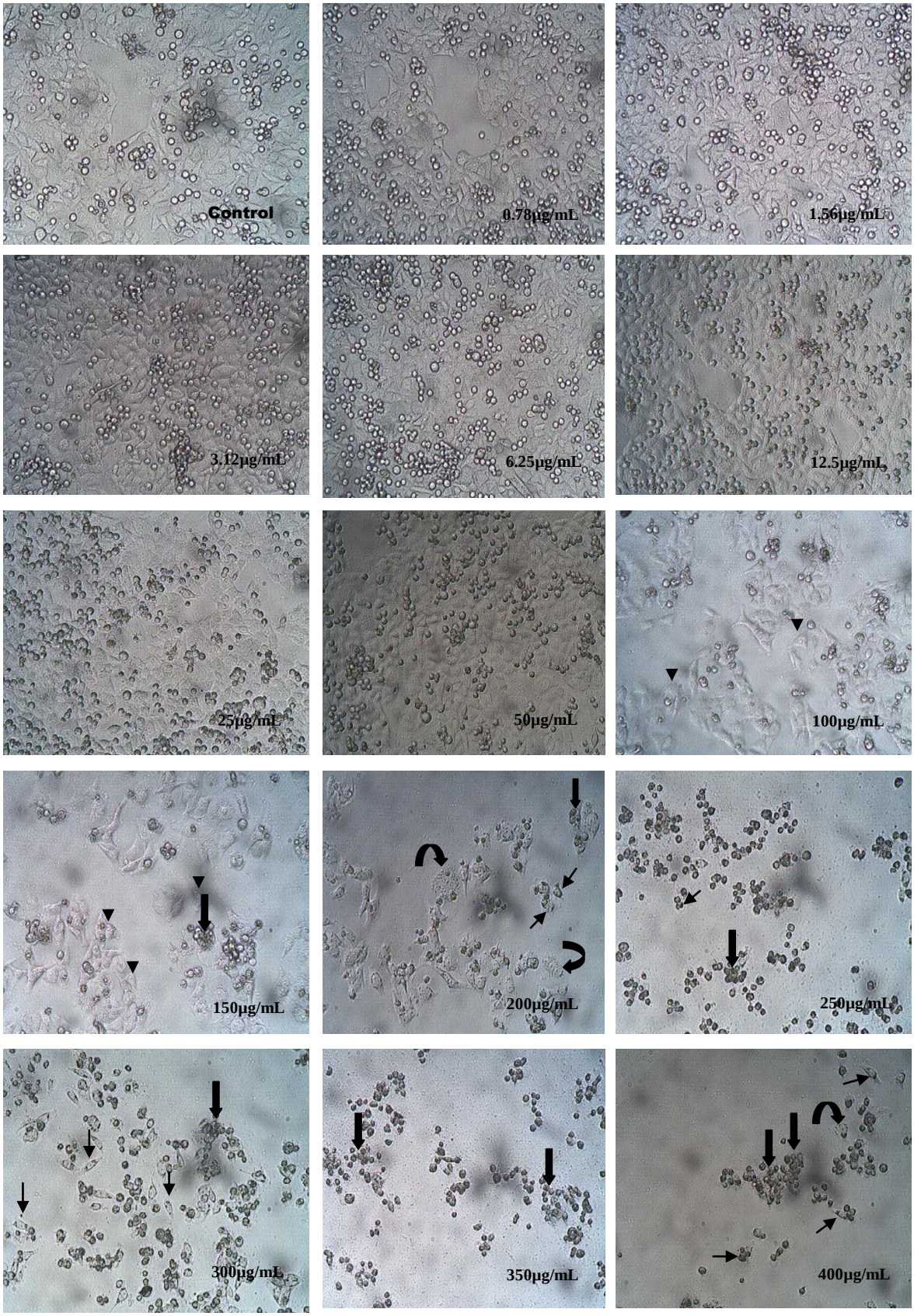

Figure 4. Phase-contrast photomicrographs of control (DMSO) and 3-hydroxycoumarin-treated HeLa cell lines. Different concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) was used. Note adherent monolayers (0.78 to 50 µg/mL). Morphological changes were observed at the highest concentrations (100 to 400 µg/mL) revealing a decreased overall cell density as well as cell clusters with condensed chromatin (arrows full), nuclear segmentation (short arrows) shrinking (arrowheads) and cellular debris (curved arrows) Magnitude of all photos: 100X.

5. Conclusion

We report the cytotoxic effect of 3-hydroxycoumarin on Vero and HeLa cell lines for the first time in the present study. The 3-hydroxycoumarin-induced toxicity and remarkable morphological changes for both cell lines were evident from 100 µg/mL. Further studies to elucidate the detailed mechanism of these effects are underway.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgements

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Manuscrito 2

Natural and Synthetic Coumarins derivatives with Potential Activity against *Leishmania (L.) amazonensis* promastigotes

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Abstract

Background

Leishmaniasis is a neglected group of emerging diseases that have been found in 98 countries and are caused by parasitic protozoa of the genus Leishmania. All the drugs currently in use as pentavalent antimonials are highly toxic with serious side effects and a prolonged treatment regimen, and there is a pressing need of new leishmanicidal compounds. Natural products have been considered good tools for prospecting of new active drugs or models for new therapeutic drugs. Coumarins are a group of natural phenolic compounds that shows several pharmacological activities and represents as target valuable molecules against several diseases. In this study, we examined the effect of coumarin and its derivatives (3- and 4-hydroxycoumarin) on *Leishmania (L.) amazonensis* growth, cytotoxicity on macrophages J774, anti-oxidant and hemolytic potential activity.

Methodology/ Principal findings

Promastigotes of *Leishmania (L.) amazonensis* were treated with different concentrations of coumarin and its derivatives (3- and 4-hydroxycoumarin) and characteristics morphological of parasites was analyzed with respect to the most effective compound by scanning electron microscopy. Cytotoxicity was measured using MTT colorimetric assay and the morphological features of these cells were evaluated by phase-contrast microscopy. Antioxidant activity was evaluated using DPPH radical scavenging. Our results demonstrated that 3-hydroxycoumarin (IC_{50} = 6.25 µg/mL) is more effective against the parasites than coumarin and 4-hydroxycoumarin (IC_{50} > 100 μg/mL). Morphological changes were observed in shape and the size of the parasite body as well as in the growth behavior of promastigotes and on macrophages J774 cell

lines under effect of 3-hydroxycoymarin. Cytotoxicity assays showed that the action of the 3-hydroxycoumarin more specific for protozoans, and it is not toxic to macrophages cell line (J774). Hemolytic tests were performed and we verified low percentage of hemolysis by 3-hydroxycoumarin (6.94-8.12%). The scavenging ability to DPPH free radicals revealed by compounds decreases in the following order: Trolox > 3-hydroxycoumarin > 4-hydroxycoumarin > coumarin.

Conclusion/ Significance

This study showed, for the first time, that 3-hydroxycoumarin significantly inhibits grownth *Leishmania (L.) amazonensis* promastigotes. Therefore, 3-hydroxycoumarin can be considered an interesting candidate for future studies regarding as a prototype drug for the treatment of leishmaniasis.

Introduction

Leishmaniasis is considered as one of the infectious parasitic diseases endemic of great relevance and a serious public health problem. According to recent report from the World Health Organization, there are three main forms of leishmaniasis: visceral (often known as “kalazar” and the most serious form of the disease), cutaneous (the most common), and mucocutaneous (also known as “espundia”, occurs years after the onset of cutaneous leishmaniasis), affecting collectively 12 million people in 98 countries, with more than 350 million people at risk [1]. Moreover, there are estimated 1.3 million new cases and 20.000 to 30.000 deaths occur annually [2]. Cutaneous leishmaniasis (CL) is caused by different species of parasites as *Leishmania (Leishmania) amazonensis* and is the most common form of leishmaniasis involving skin lesions, mainly ulcers, on exposed parts of the body, leaving life-long scars and serious disability [3]. About 75% of CL cases occur in the Americas, Mediterranean basin, Middle East and Central Asia and resides in the following ten countries: Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru [4].

This parasitic infection is transmitted to its mammal hosts, including domesticated and sylvatic animals, by the bite of infected female phlebotomine sand flies. Leishmania parasites need their vectors to complete their life cycle and to propagate. Leishmaniasis is caused by a protozoan of the Leishmania genus and presents a digenetic life cycle, with two morphological forms in their life cycle: non-motile amastigotes in the mononuclear phagocytic system of the mammalian host, and extracellular flagellated promastigotes in the digestive organs of the vector [5, 6].

Currently, there are no effective vaccines and a considerable number of drugs are used in the treatment of leishmaniasis and most of the commonly used drugs are

toxic and do not cure or eliminate the parasite, from infected individuals. Failure to treat leishmaniasis successfully is often due to increased chemoresistance of the parasite, although they are costly and require long-term treatment. These include pentavalent antimonials compounds as the first-choice drugs for treatment, Amphotericin B and paromomycin are the second-line agents. There is a pressing need for the identification of novel drug target and the development of more effective, less toxic drugs, safe and provide better outputs for the treatment of leishmaniasis [7, 8].

Previous studies have shown that various classes of natural products are promising against different species of Leishmania *in vitro*. These include saponins [9], acetogenins [10], triterpenes [11], chalcones [12], coumarins [13], flavonoids [14], quinolones [15], and alkaloids [16]. In this context, natural and synthetic coumarin derivates are considered to exhibit promising pharmacological properties that depend upon of their chemical structures and play important roles in the prospect of pharmacologically active compounds [17].

Both natural and synthetic coumarin derivatives have drawn much attention due to a wide range of biological activities, such as anti-inflammatory [18-20], anticancer [21, 22], anti-oxidant [23, 24], anti-coagulant [25], antiparasitic [26], as well as antiviral and antibacterial [27, 28]. An important class of coumarins derivates, the hydroxycoumarins, showed relevant roles in the prospection of pharmacological active compounds [29]. Hydroxycoumarins are phenolic compounds which act as potent metal chelators and free radical scavengers [30-32]. Previous studies have demonstrated that the 4-hydroxycoumarin is used as an intermediate in the synthesis of various extremely common pharmaceuticals, such as warfarin and acenocoumarol, which are used in medical practice as an anticoagulant [33], and some derivatives like 7-hydroxycoumarin

(umbelliferone) and 3-hydroxycoumarin showed (respectively) significant anti-inflammatory activity [34] and photoprotective effect [35].

Although, some studies have showed several biological activities of natural and synthetic coumarin derivatives, the activity of certain hydroxycoumarins, such as 3-hydroxycoumarin, has not been fully investigated. In this study, we have analyzed the *in vitro* activities of natural and synthetic coumarin derivates on viabilities of *Leishmania (L.) amazonensis*-promastigotes and J744 macrophage cell line, as well as characteristics morphological changes by scanning electron microscopy and phase-contrast microscopy, respectively. Additionally, we also evaluated *in vitro* hemolytic and antioxidant effects of these compounds. More studies are needed to strengthen the use of these compounds in a route that can be exploited as a potential leishmanicidal agent.

Materials and Methods

Parasites and macrophages

The *Leishmania (Leishmania) amazonensis* promastigotes (MHOM/BR/77/LTB0016) was kindly provided by Dr. Osvaldo Pompílio de Melo Neto from Department of Microbiology, Research Center Aggeu Magalhães, Pernambuco, Brazil. Parasites were maintained at 26°C, in Liver Infusion Tryptose medium (LIT, HiMedia, Laboratories Pvt. Ltda., Mumbai, India) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0,2% hemin (Sigma, St Louis, MO, USA), penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Gibco BRL, Life Technologies, Paisly, UK).

J774 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Gibco BRL, Life Technologies, Paisly, UK). The culture was maintained at 37 ºC in an atmosphere of 5% CO_2 and 95% of relative humidity.

Compounds

Coumarin and its derivatives (3-and 4-hydroxycoumarins) was kindly provided by Dr. José Maria Barbosa-Filho from Laboratory of Pharmaceutical Technology, Campus I, Federal University of Paraíba, João Pessoa, Paraíba, Brazil. Stock solutions were dissolved in dimethylsulfoxide (DMSO) and maintained at 5°C. Dilutions from the stock solutions were done in culture medium. Amphotericin B (Sigma, St Louis, MO, USA) was used as the reference standard drug.

Evaluation of *in vitro* antileishmanial activity

The 50% inhibitory concentration of *L. (L.) amazonensis* promastigotes growth (IC_{50}) was evaluated by the colorimetric method MTT [3-{4.5-dimethylthiazol-2-yl}-2.5-diphenyltetrazolium, SIGMA] that is based on the conversion of the tetrazolium salt into the colored formazan product. The promastigotes were seeded (1×10^5 cells/mL) with LIT medium in 96-well microplates for 72 h in the presence of increasing concentrations of coumarin and its derivatives (1.56 to 400 µg/mL) and Amphotericin B, which was used as a positive control (0.19 to 100 µg/mL) and LIT-DMSO (negative control). After the incubation period with coumarin and derivatives (3- and 4-hydroxycoumarins) and amphotericin B, were added to each well microplates 20 µL of MTT solution (5 mg/mL). The microplate was then incubated for 3 h at 26ºC, MTT solution was aspirated and 100 µL DMSO was added for the solubilization of the formazan crystals. After solubilization, the absorbance was measured by using a multi-well scanning spectrophotometer (SkanIt Software 2.4.5 RE for Varioskan Flash, Thermo Scientific, Massachusetts, USA) at a wavelength of 595 nm. The results were expressed as percentage of cell viability when compared with the negative control group [36]. All the experiments were performed in triplicate.

Scanning electron microscopy

For the morphological analysis, *L. (L.) amazonensis* promastigotes that were treated for 72 h at 26°C with concentrations that corresponded to the IC_{50} for 3-hydroxycoumarin (6.25 µg/mL) and Amphotericin B (3.12 µg/mL) were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde and 0.1 M of sodium cacodylate buffer (pH 7.2) for 1-3h. The parasites were rinsed in the same buffer and post-fixed in solution of 1% OsO_4 for one

hour at room temperature. After post-fixing, all samples were washed in the same buffer and dehydrated gradually increasing the ethanol concentrations (30-100%) and were critical point dried using CO_2, mounted on metal stubs, and coated with gold (5-30 nm) for observation in a scanning electron microscope (JEOL JSM T-200, Tokyo, Japan).

Cytotoxicity assay

Cell viability. It was determined using the MTT assay. Briefly, J774 macrophages ($2x10^5$cells/mL) was seeded in 96-wells plates and incubated for 24 h. 3-hydroxycoumarin was dissolved in dimethylsulfoxide and added in different concentrations (0.78-400 μg/mL). As controls, we used DMSO. The compounds dissolved were applied to culture wells in triplicate and incubated for 72 h. The formation of formazan was measured by adding 20 μL de MTT (5mg/mL) to each well and the cells were incubated at 37°C in the dark for 3h. After his time, all supernatant was discarded and subsequently the formazan crystals were dissolved in DMSO (100 μL). The optical density of each well was measured at 595 nm using a scanning spectrophotometer (SkanIt Software 2.4.5 RE for Varioskan Flash, Thermo Scientific, Massachusetts, USA). The selectivity index (SI = CC_{50}/IC_{50}) was calculated by ratio of toxicity to macrophages *vs.* toxicity to the parasites after 72 h incubation. All the experiments were performed in triplicate.

Phase-contrast microscopy. J774 macrophages were cultured in 96-well plates for 72 h, in the presence or absence of different concentrations of 3-hydroxycoumarin. After treatment, the morphological features of these cells were evaluated by phase-contrast microscopy using an inverted microscope LEICA (Leica microsystems, Wetzlar,

Germany) equipped with digital camera (MOTICAM BA 2.000, Campinas, Brazil) and the digital photographs were taken using the Motic Images Plus 2.0 software.

In vitro hemolytic analysis

Hemolytic activity was assayed according to the method described by Oliveira et al. [37]. Blood was centrifuged at 1000 x *g* and 4 °C for 10 min to separate the red cells from the plasma. The cells were washed three times with phosphate-buffered saline (PBS; pH 7.4) and again centrifuged. A 1% suspension of red blood cells was used for the test. Each tube received 1.1 mL of cell suspension and 0.4 mL of coumarin and its derivatives in the concentrations of 31.2 to 1000 µg/mL. The negative control was only solvent and the positive control received Triton X-100 (0.4 mL). After 60 min incubation at room temperature, the cells were centrifuged and the supernatant was used to measure the absorbance of the liberated hemoglobin and the magnitude of hemolysis was determined by spectrophotometer (SkanIt Software 2.4.5 RE for Varioskan Flash, Thermo Scientific, Massachusetts, USA) at 540 nm.

DPPH Radical-Scavenging Activity

The free radical-scavenging activity of the compounds (coumarin and its derivatives: 3-and 4-hydroxycoumarins) was measured in terms of hydrogen donating using the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich) described previously [38, 39]. An aliquot of 250 µL of DPPH solution (1mM) was mixed with 40 µL of different concentrations of coumarin and its derivates (31.2 to 1000 µg/mL). Thirty minutes later, the absorbance was measured at 517 nm. For the

reference compound was used 40 µL of Trolox in presence of the DPPH solution. For the blank was added 40 µL of methanol.

Statistical analysis

All values were presented as mean ± SEM from three independent experiments carried out in triplicate. The statistical differences between groups were determined by Student's *t* test. ANOVA was used to find the significance of difference between the values. *P* values < 0.05 were considered statistically significant and were displayed graphically using the computer software package Origin-Data Analysis and Technical Graphics, version 8.1 (Copyright Software, Inc.). The half maximal inhibitory concentration (IC_{50}) values were calculated by linear interpolation.

RESULTS

In vitro antileishmanial activity of coumarin and its derivatives against *L. (L.) amazonensis* promastigotes

L. (L.) amazonensis promastigotes were grown in the presence of 1.56 to 400 µg/mL of coumarin and its derivatives (3-and 4-hydroxycoumarins). Significant inhibition of parasite growth ($p<0.05$) was detected after 72 h of the treatment with 3-hydroxycoumarin (IC_{50} = 6.25 µg/mL), when compared with coumarin and 4-hydroxycoumarin that showed an IC_{50} >100 µg/mL Fig 1. The IC_{50} of Amphotericin B against *L. (L.) amazonensis* promastigotes was calculated (3.25 µg/mL) and it was in according to the value described by Colares et al. [40].

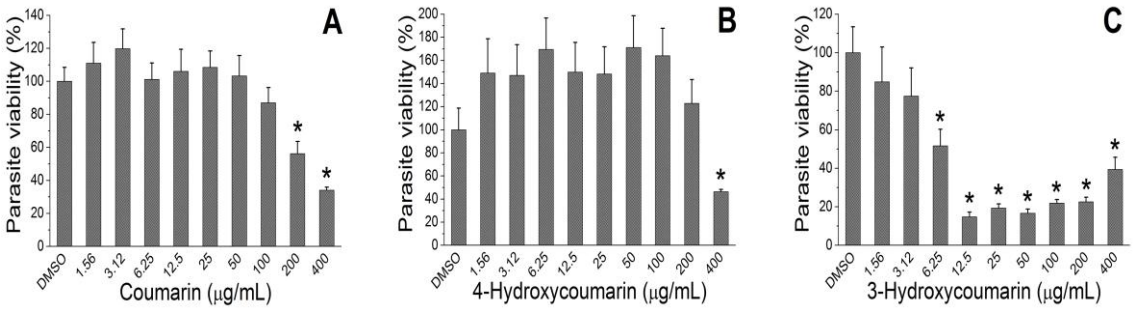
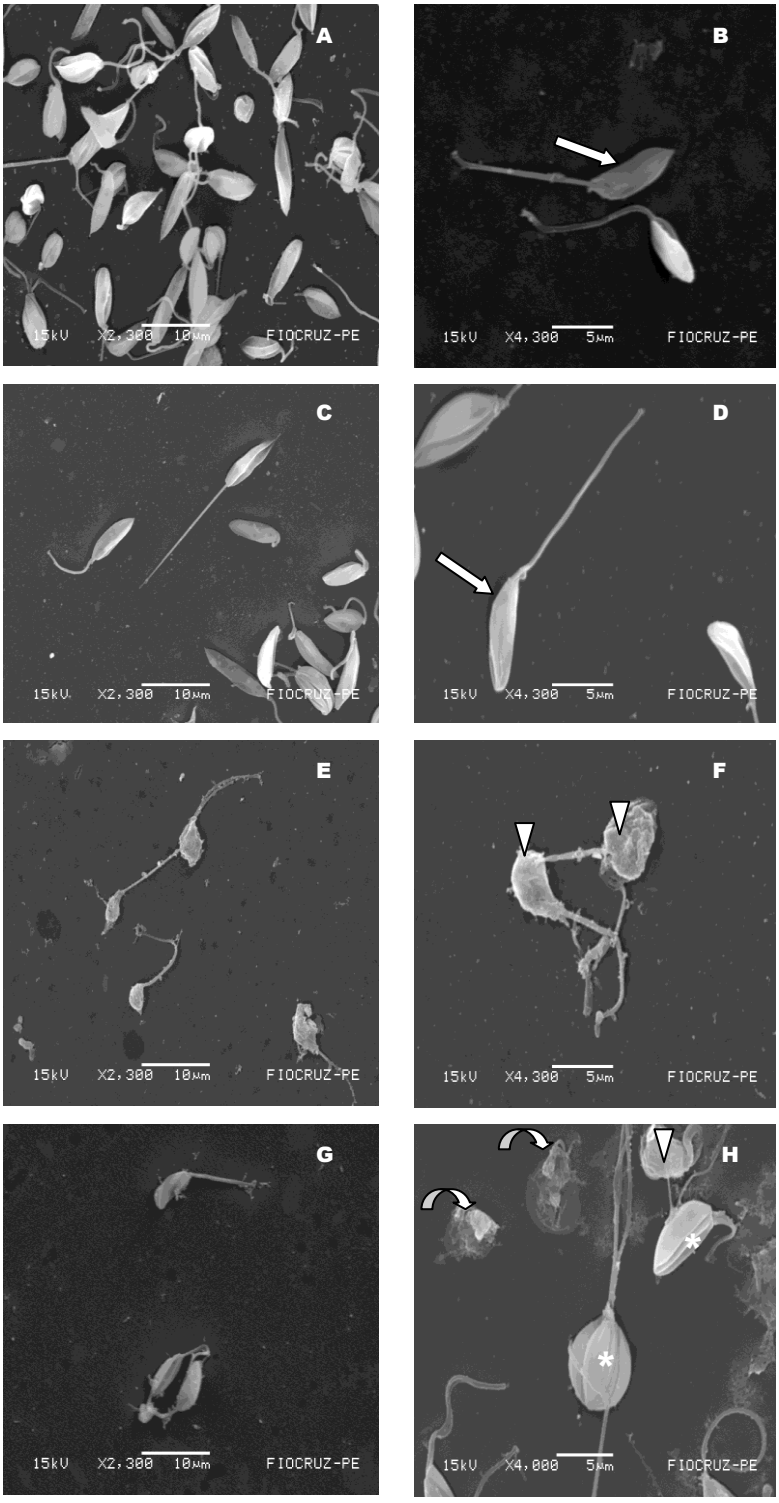


Fig 1. Effect of coumarin and its derivatives (3- and 4-hydroxycoumarins) on *Leishmania (L.) amazonensis* promastigotes growth. Parasites were treated with different concentrations (1.56 to 400 µg/mL) of coumarin and its derivatives (3- and 4-hydroxycoumarins) and the growth was estimated after 72 h (A, B and C). *P* values were obtained comparing the treated groups with control (DMSO), and asterisk symbol indicates that $p<0.05$ (ANOVA). Values represent mean ± SEM from three independent experiments carried out in triplicate.

Scanning electron microscopy of *Leishmania (L.) amazonensis* promastigotes

Scanning electron microscopy revealed that 3-hydroxycoumarin caused morphological alterations in the promastigote forms of *L. (L.) amazonensis* compared with untreated parasites cultured in the LIT Fig 2A, B and in the presence of DMSO Fig 2C, D, that showed typical characteristics, with an elongated shape and free flagellum. We observed alterations in shape and size and cellular disintegration in 3-hydroxycoumarin-treated parasites. Fig 2G, H, showed that these alterations were more pronounced in parasites treated with the IC_{50} (6.25 µg/mL) of 3-hydroxycoumarin. Thus promastigotes treated with amphotericin Fig 2E, F when compared with the treatment of 3-hydroxycoumarin; in both conditions, we observed alterations in shape and the size of protozoan such as shortening of the parasite body, protrusions and ruffling of the membrane.



278 **Fig 2. Scanning electron micrograph (SEM) of *Leishmania (L.) amazonensis* displaying the**
279 **characteristic morphology of promastigotes forms.** The electron micrographs illustrate the
280 morphological characteristics of control promastigotes in LIT (A, B) and in DMSO (C, D), we
281 note elongated body and emerging flagellum (white arrows). Promastigotes treated with IC_{50}
282 values of amphotericin (E, F) and of 3-hydroxycoumarin (G, H) showed alterations in shape and
283 the size of protozoan such as shortening of the parasite body (arrowheads), protrusions and
284 ruffling of the membrane (asterisks), and cellular disintegration (curved arrows).
285 Magnifications: X 2.300 (A, C, E and G), X 4.300 (B, D and F), X 4.000 (H).
286

The growth behavior of *Leishmania (L.) amazonensis* promastigotes were assessed by scanning electron microscopy (SEM). We observed that *L. (L.) amazonensis* promastigotes have exponential growth in culture progress through a range of morphologies which make up a single cell cycle, where it is possible to note Fig 3A, B and C. Under effect of 3-hydroxycoumarin (IC_{50}) we observed significant reduction in the density of parasites and rosette formations (stars) Fig 3D, E and F.

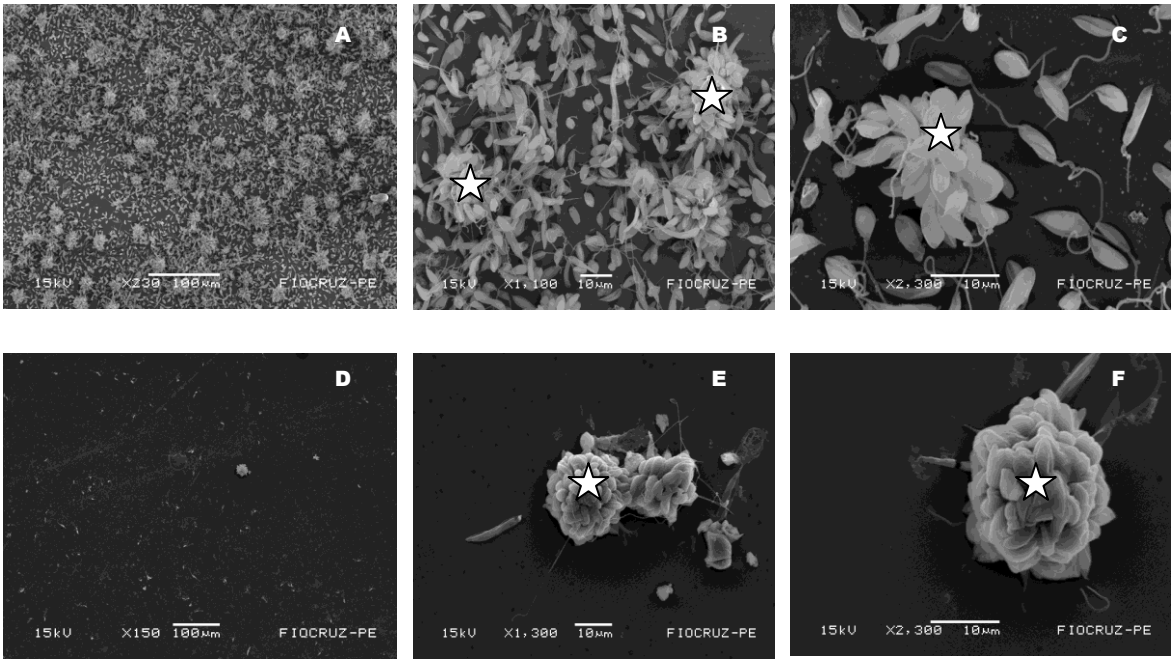


Fig 3. Growth behavior of *Leishmania (L.) amazonensis* promastigotes in culture by Scanning electron micrograph (SEM). Scanning electron micrograph displaying the exponential growth of *L. (L.) amazonensis* promastigotes in control culture (DMSO) revealing a range of morphologic characteristics (A, B and C). Under effect of 3-hydroxycoumarin (IC_{50}) we observed significant reduction in the density of parasites and rosette formations (stars) (D. E and F). Magnifications: X 230 (A), X 1.100 (B), X 150 (D), X 1.300 (E) and X 2.300 (C and F).

In vitro cytotoxic effect of 3-hydroxycoumarin on J774 macrophages

The cytotoxicity of 3-hydroxycoumarin on J774 macrophages was determined by the MTT method with 72h. The percentage viability of these cells was established according to the concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) of 3-hydroxycoumarin. The lowest concentrations of 3-hydroxycoumarin (0.78 to 50 µg/mL) exhibited viability higher than those of the control

306 (DMSO) and higher concentrations (100 to 400 µg/mL) were ineffective in preventing
307 the loss of cell viability Fig 4 B. Our results were statistically significant (p<0.05) when
308 compared to control (DMSO). The results obtained in this work, suggested that the 3-
309 hydroxycoumarin could promote or inhibit the viability of normal cells in a
310 concentration-dependent manner.

311 The effect of different concentrations of 3-hydroxycoumarin on strain of
312 macrophages (J744) is illustrated in Figure X. Cells were cultivated as adherent
313 monolayer at lower concentrations of 3-hydroxycoumarin (0.78 to 50 µg/mL), identical
314 like those observed in control. Morphological changes Fig 4C included retraction of
315 cytoplasmic extensions that was observed from the concentration of 100 µg/mL.
316 Moreover, especially at the highest concentrations (250 to 400 µg/mL), dramatic
317 changes in macrophages morphological features was observed revealing a decreased
318 overall cell density as well as cell shrinking and dense protrusions burgeon at the cell
319 margins. The cells demonstrated failure to reestablish intercellular associations and the
320 growth pattern as adherent monolayer, due the reorganization of the cytoskeletal
321 network caused by oxidative injury-induced actin remodeling.

322

323

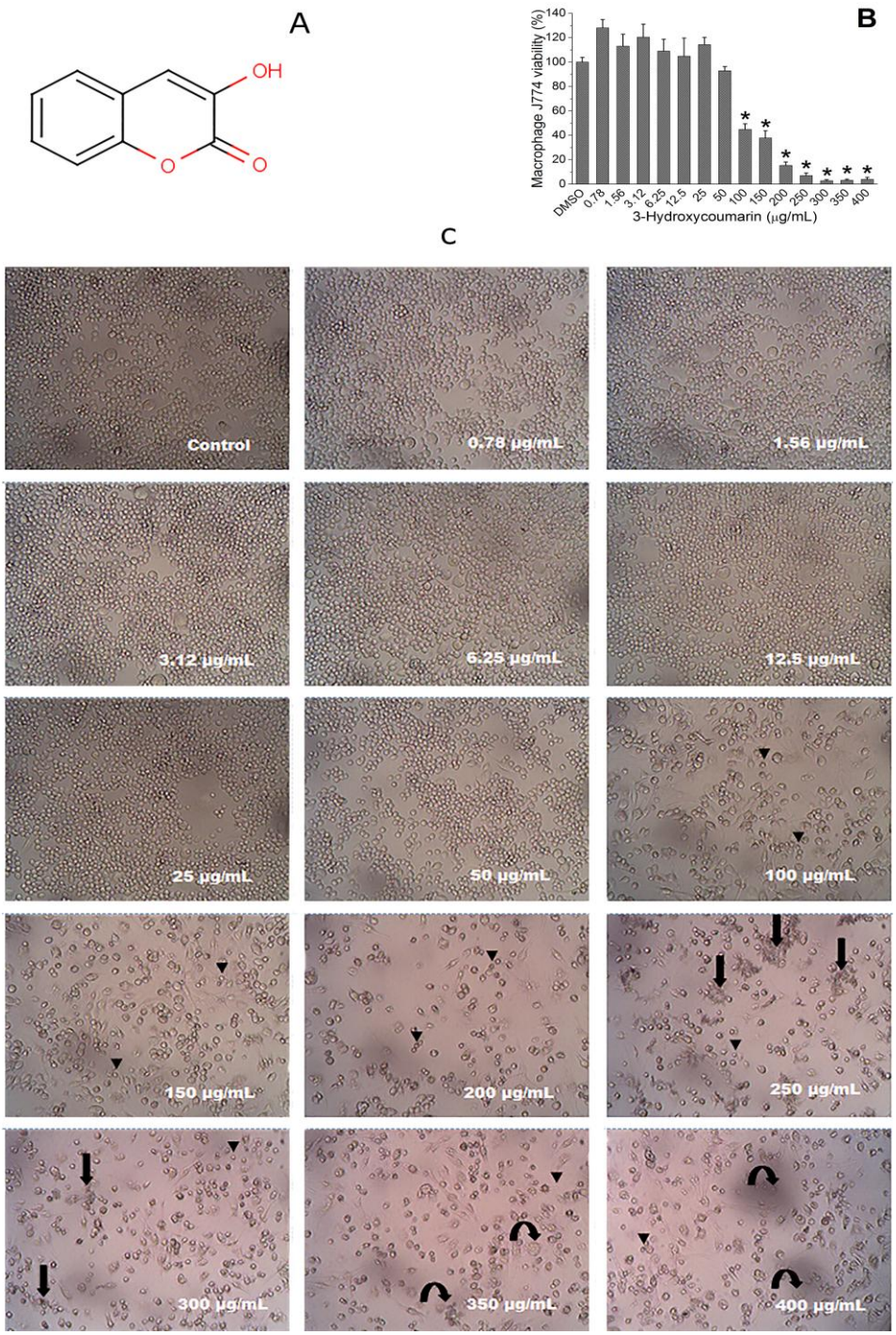


Fig 4. *In vitro* cytotoxic effect of 3-hydroxycoumarin on J774 macrophage cell line. The figure shows the chemical structure of 3-hydroxycoumarin (A). *In vitro* cytotoxicity of 3-hydroxycoumarin in different concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) on J774 macrophages was evaluated (B). *P* values were obtained comparing the treated groups with control (DMSO), and asterisks symbol indicates that $p<0.05$ (ANOVA). Values represent mean ± SEM from three independent experiments carried out in triplicate (B). Phase-contrast photomicrographs of control (DMSO) and 3-hydroxycoumarin-treated macrophages were presented. We noted adherent monolayers (0.78 to 50 µg/mL) and morphological changes were observed at the highest concentrations (100 to 400 µg/mL) revealing a decreased overall cell density as well as cell shrinking (arrowheads), dense protrusions burgeon at the cell margins (arrows full), and cellular debris (curved arrows) (C).

The CC_{50} of 3-hydroxycoumarin against J774 macrophages was 100 µg/mL. A ratio of cytotoxicity to biological activity (CC_{50} macrophages/IC_{50} promastigotes) was used to determine the selectivity index (SI) of 3-hydroxycoumarin (**Table 1**). We found the value of SI=16, indicating lower toxicity of 3-hydroxycoumarin.

Table 1. Leishmanicidal and cytotoxic activity of coumarin and its derivatives.

Compounds	Promastigotes IC_{50} (µg/mL)	Macrophages[a] CC_{50} (µg/mL)	Selectivity index[b] (SI)
Coumarin	> 400	nd[c]	nd[c]
3-hydroxycoumarin	6.25	100	16
4-hydroxycoumarin	400	nd[c]	nd[c]

[a] J774 macrophages.
[b] SI= CC_{50} macrophages/ IC_{50} promastigotes.
[c] nd= not determined.

Hemolytic activity of coumarin and its derivatives

Table 2 showed that 4-hydroxycoumarin in accordance with the concentrations tested (31.25 – 1000 µg/mL) presented high rate of hemolysis when compared with the values of coumarin and 3-hydroxycoumarin. The low percentage of hemolysis of 3-hydroxycoumarin (6.94 - 8.12%) corroborate with the results of cell cytotoxicity, proving to be more specific as possible antileishmanial agent.

Table 2. Hemolytic activity of coumarin and its derivatives

	Hemolysis (%)		
Concentrations (µg/mL)	**Coumarin**	**3-hydroxycoumarin**	**4-hydroxycoumarin**
31.25	7.05 ± 0.43	6.94 ± 0.48	7.39 ± 0.75
62.5	7.39 ± 0.18	7.12 ± 0.60	8.20 ± 0.79
125	7.88 ± 0.71	7.36 ± 0.69	8.37 ± 0.60
250	7.91 ± 0.21	7.56 ± 0.51	8.89 ± 1.97
500	7.98 ± 0.42	7.91 ± 0.0	26.75 ± 3.37
1.000	8.05 ± 0.37	8.12 ± 0.18	97.17 ± 15.82

All values are means ± SD (n=3).

Antioxidant activity of coumarin and its derivatives

The results are summarized in **Table 3** and showed the indices of reduction of the radical scavenging activity of coumarin and its derivatives (3- and 4-hydroxycoumarins) at different concentrations (31.25-1.000 µg/mL). The data obtained clearly indicate the concentration-dependent activities when compared with Trolox, as a reference compound. The scavenging ability to DPPH free radicals revealed by compounds increases in the following order: Trolox > 3-hydroxycoumarin > 4-hydroxycoumarin > coumarin.

Table 3. Profile of DPPH radical scavenging activity of coumarin and its derivatives

	DPPH[a] radical scavening activity (%)			
Concentrations (µg/mL)	**Coumarin**	**3-hydroxycoumarin**	**4-hydroxycoumarin**	**Trolox[b]**
31.25	0.53 ± 0.29	28.52 ± 0.92	7.98 ± 0.62	nd[c]
62.5	1.53 ± 0.52	38.84 ± 2.07	13.47 ± 0.72	56.14
125	3.34 ± 0.58	51.08 ± 1.89	17.15 ± 0.50	95.73
250	4.68 ± 3.97	63.26 ± 1.71	23.75 ± 1.79	96.02
500	5.21 ± 0.17	73.01 ± 0.82	37.08 ± 0.43	96.66
1000	7.84 ± 3.23	82.27 ± 0.36	44.24 ± 2.73	nd[c]

[a] 2,2-Diphenyl-1-picrylhydrazyl.
[b] Reference compound.
[c] nd - not determined.
All values are means ± SD (n=3).

Discussion

One of the aim objectives of this *in vitro* study was to evaluate the potential activity of natural and synthetic coumarin derivatives against *Leishmania (L.) amazonensis* promastigotes. Additionally, experiments were made to investigate cell citotoxicity on J774 macrophage cell line as well as hemolytic and anti-oxidant activities. In the context, biological activities of coumarins have becoming relevant in recent studies due its different effects to diseases and less damage to normal cells [41]. Our results showed the potential growth inhibition of *L (L.) amazonensis* promastigotes by 3-hydroxycoumarin. This compound caused significant decreases in promastigotes number at concentration of 6.25 µg/mL (IC_{50} value), when compared with the action of amphotericin B (IC_{50}=3.12 µg/mL).

Promastigotes of *L. (L.) amazonensis* treated with the IC_{50} of 3-hydroxycoumarin showed different degrees of morphological changes like alterations in

391 shape and the size of protozoan such as shortening of the parasite body, protrusions and
392 ruffling of the membrane and this finding has been previously shown for apoptotic-like
393 death induced by distinct compounds [42]. The growth behavior of Leishmania *(L.)*
394 *amazonensis* promastigotes were also assessed by scanning electron microscopy (SEM)
395 and we observed that the parasites have exponential growth in culture progress
396 organized in rosettes and this formation were reduced when promastigotes were
397 cultured in the presence of 3-hydroxycoumarin harming a genuine stage in the life cycle
398 of these parasites [43]. Therefore, coumarins derivatives have received increasing
399 attention for the wide biological and pharmacological activities demonstrating
400 therapeutic potential [44].

401 Cytotoxicity assays showed that the action of the 3-hydroxycoumarin more
402 specific for protozoans, and it is not toxic to macrophages cell line (J774). The
403 protective effect may be related to antioxidant activity of coumarin derivatives. The
404 antioxidant activity of phenolic compounds is due to their ability to scavenge free
405 radicals, donate hydrogen atoms or electron, or chelate metal cations. This family of
406 compounds acts as antioxidants and thereby protect from degenerative diseases in which
407 reactive oxygen species (ROS) are involved [45-46]. We also denote morphological
408 changes with high concentrations of 3-hydroxycoumarin (from 100 µg/mL), the cells
409 demonstrated failure to reestablish intercellular associations and the growth pattern as
410 adherent monolayer, due the reorganization of the cytoskeletal network possible caused
411 by oxidative injury-induced actin remodeling. It has been reported that deleterious
412 effects of ROS on human cells may end in oxidative injury leading to programmed cell
413 death [47].

414 Coumarins possess a great structural diversity, since the replacements can occur
415 at any of the six available sites of their basic molecular moiety (1,2-benzopyrone) and

according to recent studies the presence of substituents groups is important for the potency of cytotoxicity [48, 49]. These finding relate the hydroxyl or methoxy groups increased the affinity of coumarin with its molecular target, refining its cytotoxic effects [50]. Hemolytic tests were performed and we verified low percentage of hemolysis by 3-hydroxycoumarin (6.94-8.12%). Ours results corroborate also with those related to concentration-dependent cellular cytotoxicity of 3-hydroxycoumarin, proving to be more specific as possible antileishmanial agent. Furthermore, the hemolytic potential can be used to indicate the toxicity of molecules on erythrocytes that could compete with water-mediated intermolecular hydrogen binding between the lipid bilayer and weakening the membrane [51, 52].

Additionally, we also investigated the antioxidant activity of coumarin and its derivatives at concentrations (31.2-1.000 µgmL). The scavenging ability to DPPH free radicals revealed by compounds studied followed the order: Trolox > 3-hydroxycoumarin > 4-hydroxycoumarin > coumarin. The significant radical-scavening activity of 3-hydroxycoumarin has been reported in this study. Recent data showed that the presence of hydroxyl groups at 3 and 5 position of the basic molecule may influence the structure-related biological activities of coumarin [53, 54].

We have reported the cytotoxic activity of natural and synthetic coumarins derivatives against *Leishmania (L.) amazonensis* promastigotes and for the first time we highlight the effect of 3-hydroxycoumarin as possible leishmanial agent. The results suggest that this compound can be used as promising prototype for drug development against Leishmaniasis and further research will be required in order to understand the probable mechanisms of action.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: EBO, JMBF, ECS, PLM. Performed the experiments: EBO, JSBL, GDMS, LCA, FAB, RJRP, TFS, LLSS, DCM. Analyzed the data: EBO, LCA, FAB, TFS, CGR, ECS, PLM. Contributed reagents/materials/analysis tools: EBO, DCM, JMBF, CGR, PML. Wrote the paper: EBO, PLM.

6. CONCLUSÕES

A 3-hidroxicumarina não foi citotóxica para as células J774, Vero e HeLa nas concentrações de 0,78 a 50 µg/mL;

- Alterações morfológicas nas células J774, Vero e HeLa foram visualizadas a partir da concentração de 100 µg/mL de 3-hidroxicumarina;

- A 3-hidroxicumarina foi a mais eficiente contra a forma promastigota da *Leishmania (L.) amazonensis* quando comparadas a cumarina e a 4-hidroxicumarina;

- Alterações da morfologia dos parasitas e do comportamento do crescimento celular foram observados em microscopia eletrônica de varredura com a IC_{50} obtida experimentalmente da 3-hidroxicumarina e do controle positivo (Anfotericina B);

- A capacidade de eliminir o radical DPPH aumenta na seguinte ordem: Trolox> 3-hidroxicumarina > 4-hidroxicumarina >cumarina e a 3-hidroxicumarina obteve a melhor atividade antioxidante quando comparadas aos demais compostos testados.

- A 3-hidroxicumarina mostrou baixo percentual de hemólise (6,94% - 8,12%).

- Os resultados obtidos sugerem que a 3-hidroxicumarina pode ser usado como um promissor protótipo para o desenvolvimento de novas drogas para a Leishmaniose.

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8. ANEXOS

8.1. Guia para autores de manuscritos submetidos a Periódicos cadastrados pelo Qualis Capes 2014

MANUSCRITO 1

Revista: Evidence-BasedComplementaryandAlternative Medicine (eCAM)

Área de avaliação: Farmácia

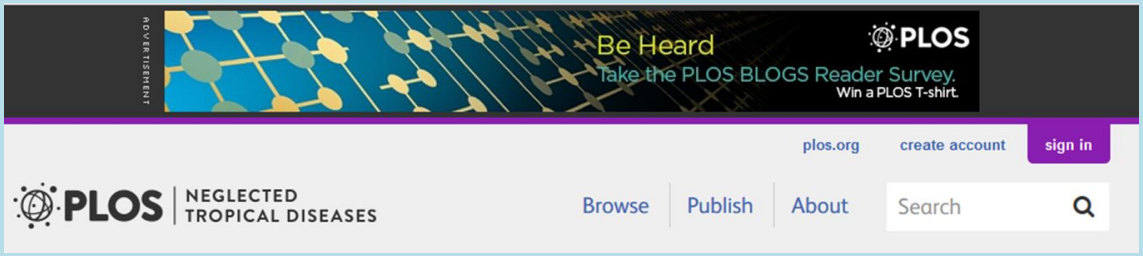
Classificação: B2

MANUSCRITO 2

Revista: PLOS Neglected Tropical Diseases

Área de avaliação: Farmácia

Classificação: A1



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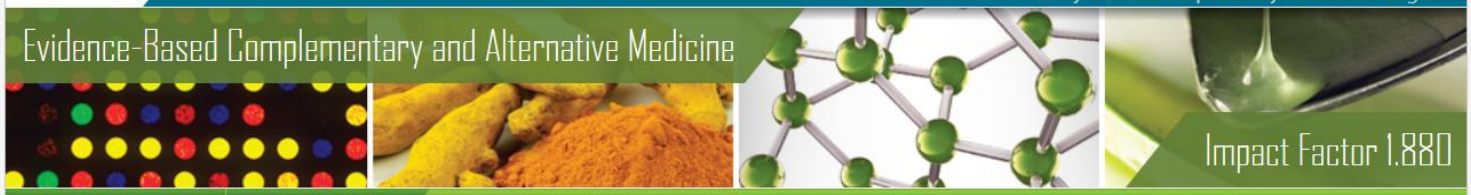

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Journal	Evidence-Based Complementary and Alternative Medicine
Issue	Regular
Manuscript Number	9734832 (Research Article)
Submitted On	2016-01-22
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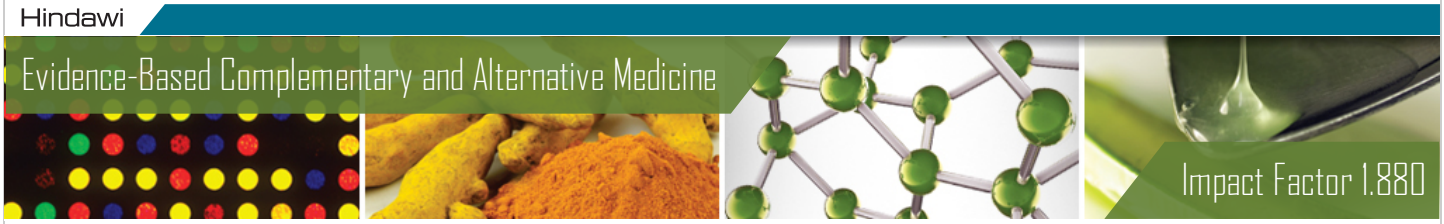

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- Full author names
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This should clearly explain the main conclusions of the work highlighting its importance and relevance.

Acknowledgments

All acknowledgments (if any) should be included at the very end of the paper before the references and may include supporting grants, presentations, and so forth.

References

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PLOS Neglected Tropical Diseases publishes original research articles of importance to the NTDs communit and the wider health community. We will consider manuscripts of any length; we encourage the submission of both substantial full-length bodies of work and shorter manuscripts that report novel findings that might be based on a more limited range of experiments.

The writing style should be concise and accessible, avoiding jargon so that the paper is understandable for readers outside a specialty or those whose first language is not English. Editors will make suggestions for hov to achieve this, as well as suggestions for cuts or additions that could be made to the article to strengthen the argument. Our aim is to make the editorial process rigorous and consistent, but not intrusive or overbearing. Authors are encouraged to use their own voice and to decide how best to present their ideas, results, and conclusions.

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- Title
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- Introduction
- Methods
- Results
- Discussion
- Acknowledgments
- References
- Supporting information Captions

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Short title	70 characters	State the topic of the study	Cigarette Smoke Exposure and Innate Immunity SODIS and Childhood Diarrhoea

Titles should be written in title case (all words capitalized except articles, prepositions, and conjunctions). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

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Source	Format
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Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
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Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013. Database: figshare [Internet]. Accessed: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214.
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Abstract

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Results

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$$p^2 + 2pq + q^2 = 1 \qquad (1)$$

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Chemical 2	Reaction 2W	Reaction 2X	Reaction 2Y	Reaction 2Z
Chemical 3	Reaction 3W[a]	Reaction 3X	Reaction 3Y[b]	Reaction 3Z
Chemical 4	Reaction 4W	Reaction 4X	Reaction 4Y	Reaction 4Z
Chemical 5	Reaction 5W	Reaction 5X	Reaction 5Y	Reaction 5Z

This is the Table 1 legend.
[a]Table footnotes belong here.
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References

1. Doe J, Data A, van Stats J, Testperson M, Ribosome D Jr, McBio GHT, et al. This is the article title. PLoS Negl Trop Dis. 2014 December 18; 8(12).
2. Doe J, Data A, van Stats J, Testperson M, Ribosome D Jr, McBio GHT. Intraspecific competition for food resources in desert-dwelling pikas. PLoS Negl Trop Dis. 2014 December 11; 8(12).
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