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**MESTRADO EM BIOQUÍMICA**

# EFEITO DO ÁCIDO BARBÁTICO DE *Cladonia salzmannii* (Nyl.) NANOENCAPSULADO SOBRE *Trypanosoma cruzi*

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**Recife, 2007**

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# EFEITO DO ÁCIDO BARBÁTICO DE *Cladonia salzmannii* (Nyl.) NANOENCAPSULADO SOBRE *Trypanosoma cruzi*

**Dissertação apresentada para o cumprimento das exigências para obtenção do título de mestre em bioquímica pela Universidade Federal de Pernambuco.**

**Ribeiro, Paula Roberta Tabosa Hirakawa**
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Ata da defesa de dissertação da Mestranda **Paula Roberta Tabosa Hirakawa Ribeiro,** realizada em 28 de fevereiro de 2007, como requisito final para obtenção do título de Mestre em Bioquímica.

Às 17:45 minutos do dia 28 de fevereiro de 2007, foi aberto, no Auditório Prof. Marcionilo Lins - Depto. de Bioquímica/UFPE, o ato de defesa de dissertação da mestranda **Paula Roberta Tabosa Hirakawa Ribeiro,** aluna do Curso de Mestrado em Bioquímica/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. Vera Lúcia de Menezes Lima, Coordenadora do Curso supra citado, fez a apresentação da aluna, de sua orientadora, Profa. Dra. Nereide Stela Santos Magalhães, de seus Co-Orientadores Profa. Dra. Regina Célia Bressan Queiroz de Figueiredo, do Depto. de Biologia Celular e Ultraestruturas, do Aggeu Magalhães/UFPE, e o Prof. Dr. Nicácio Henrique da Silva, do Depto. de Bioquímica/UFPE, e da Banca Examinadora composta pelos professores doutores: Nereide Stela Santos Magalhães, na qualidade de Presidente, Vera Lúcia de Menezes Lima, Maria das Graças Carneiro da Cunha, as três do Depto. de Bioquímica, e Janaína Campos de Miranda, do Depto. de Biologia Celular e Ultraestruturas, do Aggeu Magalhães/UFPE. Após as apresentações, a Sra. Presidente convidou a aluna para a apresentação de sua dissertação intitulada: **"Efeito do ácido barbático de *Cladonia Salzmannii* (Nyl.) nanoencapsulado sobre *Trypanosoma cruzi*"**, e informou que de acordo com o Regimento Interno do Curso, a candidata disporia de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pela aluna para responder às perguntas seria de 30 (trinta) minutos. A aluna procedeu a explanação e comentários acerca do tema em 40 (quarenta) minutos. Após a apresentação da mestranda, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, a Profa. Dra. Janaína Campos de Miranda, em seguida para a Profa. Dra. Vera Lúcia de Menezes Lima, e finalmente para a Profa. Dra. Maria das Graças Carneiro da Cunha, as quais agradeceram o convite, fizeram alguns comentários e sugestões. Ao final de suas respectivas argüições, as referidas professoras deram-se por satisfeitas. Em seguida, a Sra. Presidente usou da palavra para tecer alguns comentários, agradecer à Banca Examinadora e parabenizar a candidata. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 28 de fevereiro de 2007.

Vera Lucia de Menezes Lima.

Maria das Graças Carneiro da Cunha

Nereide Stela Santos Magalhães

Janaina Campos de Miranda

**“Sejamos sempre gratos às pessoas porque tudo o que conquistamos, nunca o fazemos sozinhos”.**

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**LISTA DE ABREVIATURAS**

***Trypanosoma cruzi*** - *T. cruzi*

**BARB -** Ácido barbático

**BARB-NC** – Nanocápsulas contendo ácido barbático

**PLGA** – Copolímero de ácido poli – láctico – glicólico

**DMSO** – dimetilsulfóxido

**LIT** – liver infusion medium

**CI $_{50}$ –** concentração que inibe 50%

**FDA** - Food and Drug Administration

**PLA** – Polímero de ácido láctico

**PCL** – Polímero de ß-caprolactona

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**RESUMO**

O objetivo deste trabalho foi avaliar os efeitos do ácido barbático (BARB) na viabilidade celular, no crescimento e na ultra-estrutura da forma epimastigota do *Trypanosoma cruzi*, o agente etiológico da doença de Chagas.

A fim de aumentar a biodisponibilidade do ácido barbático, este composto foi incorporado a nanocápsulas (BARB-NC) de ácido poli-láctico- glicol (PLGA). O crescimento e a viabilidade celular foram avaliados através da contagem na câmara de Neubauer nos tempos 24, 48 e 72 horas. Os parasites foram incubados a diferentes concentrações de BARB e BARB-NC por 72 horas após 24 horas de cultivo. Parasitas cresceram em meio livre de droga que foi usado como controle. A incubação dos parasites com nanocápsulas vazias não resultaram em atividade inibitória quando comparados ao controle, embora mudanças estruturais terem sido verificadas. Tratamento dos parasitas tanto com BARB e BARB-NC resultaram em uma inibição do cresciemtno dose-dependente. Verificou no BARB uma inibição de apenas 32% nas primeiras 24 horas com a concentração de 62,5 μg/ml. No entanto, o valor da $IC_{50}$ da BARB-NC nas 24h foi 24.66 μg/ml. A incorporação do BARB dentro de nanocápsulas potencializou o efeito tripanosomicida deste. Embora, o tratamento com BARB e BARB-NC cause trágicas mudanças morfológicas, essas alterações foram mais severas nos parasites tratados com BARB-NC.

Palavras- chaves: ácido barbático, *Trypanosoma cruzi*, nanocápsulas.

**ABSTRACT**

The aim of this study was to evaluate the effects of barbatic acid (BARB) on the viability, growth and ultrastructure of *Trypanosoma cruzi* epimastigote forms, the ethiologic agent of Chagas' disease. In order to improve the BARB bioavailability this compound was incorporated into D,L-(lactide-glycolide) copolymer (PLGA) nanocapsules (BARB-NC). The cell growth and viability was evaluated by cell counting using Neubauer chamber for 24, 48 and 72 hours. The parasites were incubated at different BARB or BARB-NC concentrations for 72 hours estimated after 24 hours of cultivation. Parasites grown in drug free medium were used as a control. The incubation of parasites with unloaded nanocapsules resulted in no inhibitory activity in comparasion with control cells, but slight ultrastructural changes were verified. Treatment of parasites with both BARB and BARB-NC resulted in a dose-dependent growth inhibition. BARB caused an inhibition of only 32% of parasite proliferation at 62.5 μg/ml at 24 hours. However, the $IC_{50}$ value of BARB-NC at 24 hours was 24.66 μg/ml. The incorporation of BARB into nanocapsules enhanced therefore its trypanocidal activity. Nevertheless the treatment of epimastigotes with both BARB and BARB-NC caused drastic morphological changes with a number of affected cells and severe ultra-structural alterations considerably higher in BARB-NC treated parasites.

Keywords: barbatic acid, *Trypanosoma cruzi*, nanocapsules.

# INTRODUÇÃO

## 1. REVISÃO DA LITERATURA

### 2.1 Sistema de Liberação Controlada de Fármacos

O sistema de liberação controlada de fármacos é o campo da atividade científica relacionado com a modulação, no tempo e no espaço, dos efeitos biológicos de drogas administradas. É constituído de vetores capazes de otimizar a velocidade de liberação e a dosagem destas. Esses carreadores, muitas vezes, têm em sua constituição polímeros biodegradáveis, que facilitam o transporte das drogas nos fluidos biológicos. Alguns polímeros que são aprovados pela Food and Drug Administration (FDA) são poli (ácido láctico) (PLA), poli (ácido láctico-co-ácido glicólico) (PLGA), poli (ε-caprolactona) (PCL). Além disso, modificam a farmacocinética e aumentam a biodisponibilidade de muitas substâncias ativas (SOPPIMATH *et al., 2001*).

A farmacocinética de droga administrada por meio de sistema de liberação controlada se mantém em concentração sanguínea constante em função do tempo (Figura 1). O que faz com que o fármaco se mantenha na faixa terapêutica, sem oscilações entre os níveis tóxicos e sub-terapêuticos, diferente dos fármacos tradicionais (BARRAT, 2000).

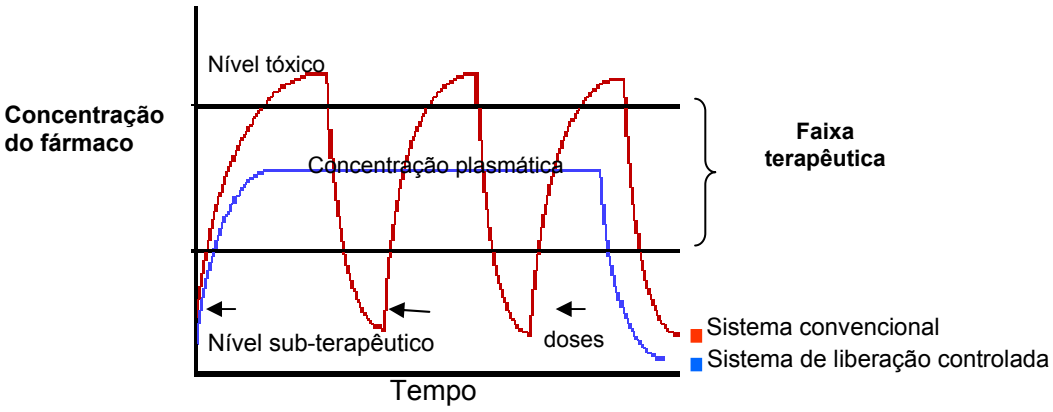


Figura 2.Comparação entre os perfis de cinética de fármacos em sistemas de liberação controlada e convencional.

FONTE: http://www.qmc.ufsc.br/qmcweb/images/drug_cinetica.jpg

De acordo com SANTOS-MAGALHÃES, *et al* (2000), o uso de nanotecnologia tem como objetivo fornecer modelos de carreadores estáveis que tenham uma boa absorção, liberação controlada e especificidade do fármaco no tecido alvo. Na preparação das nanopartículas, é necessário utilizar materiais que sejam biocompatíveis e biodegradáveis e seu tamanho não pode ultrapassar um micrometro. A droga fica retida na matriz polimérica ou no núcleo dependendo da sua natureza (FESSI *et al.,* 1989; SOPPIMATH *et al.*, 2001).

*2.1.1 Nanopartículas*

As nanopartículas são carreadores coloidais poliméricos sólidos que se apresentam sob dois tipos: nanoesferas e nanocápsulas, as quais diferem entre si segundo a composição e organização estrutural (Figura 2). As nanoesferas são formadas por uma matriz polimérica onde o fármaco fica retido ou adsorvido. As nanocápsulas são constituídas por um envoltório polimérico pouco espesso disposto ao redor de um núcleo oco ou de natureza oleosa e estabilizada por um filme interfacial de agentes tensoativos (SOPPIMATH *et al.*, 2001). O tamanho médio das nanocápsulas varia de 50 a 400 nm.

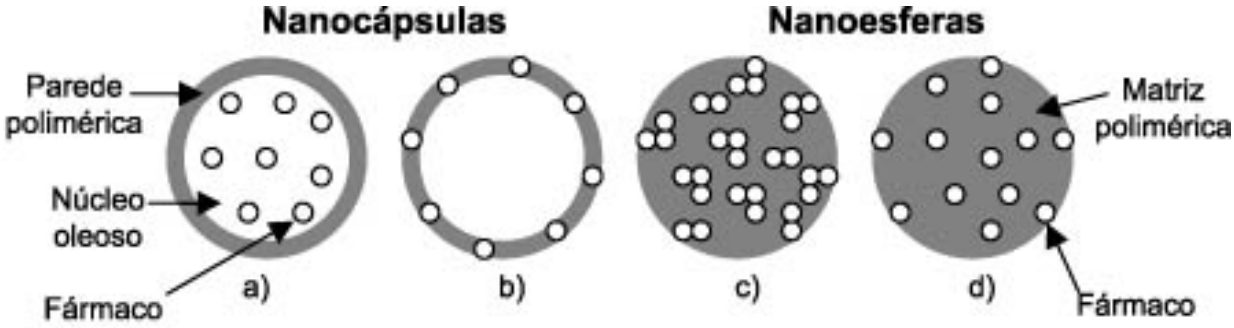


Figura 2. Representação esquemática das nanocápsulas e nanoesferas.

FONTE: Schaffazick *et al.* 2003.

Um método bastante utilizado para a preparação de nanocápsulas é por deposição interfacial do polímero (FESSI *et al.,* 1989). Nesta metodologia observam-se duas fases, uma fase orgânica e outra aquosa. A fase orgânica é constituída pelo polímero, o princípio ativo, e um tensoativo lipofílico (óleo) todos diluídos em acetona. Já a fase aquosa é constituída de um tensoativo hidrofílico diluído em tampão fosfato pH=7.4. A fase orgânica é misturada lentamente à fase aquosa e desta forma, as nanocápsulas são formadas. O uso dos tensoativos, tanto lipofílico quanto hidrofílico, tem a finalidade de evitar a sedimentação das partículas e de formar partículas mais homogêneas e menores, respectivamente. Fármacos lipofílicos apresentam solubilidade na matriz polimérica e na cavidade oleosa das nanocápsulas o que leva a uma incorporação mais rápida quando comparados aos hidrofílicos (BARRAT, 2000).

A incorporação do fármaco nas nanocápsulas pode se dar por três maneiras: diretamente na cavidade interna, retido na parede polimérica ou adsorvido na superfície. Esses sistemas vêm sendo desenvolvidos para diversas aplicações terapêuticas, como por exemplo, para fármacos anticancerígenos (SANTOS *et al.*, 2006) e antibióticos (PINTO-ALPHANDARY *et al.*, 2000; DUTT & KHULLER, 2001).

Os fármacos nanoencapsulados podem ter uma liberação sítio-específica para o sistema linfático, o baço, o cérebro, os pulmões, o fígado e para uma circulação sistêmica prolongada (HANS & LOWMAN, 2002). Além disto, as nanopartículas podem ultrapassar a barreira hematoencefálica, vantagem que pode beneficiar os fármacos usados para o sistema nervoso central.

Embora sua produção seja complexa e onerosa, há diversas vantagens que justificam o uso dos carreadores de fármacos, tais como a distribuição mais seletiva nas células alvos, a diminuição do índice terapêutico o que leva também a uma redução dos possíveis efeitos colaterais dos mesmos (LANGER, 2000), proteção contra degradação da droga no trato digestivo e preservação da atividade do princípio ativo (BARRAT, 2000; WHELAN, 2001).

### *2.1.2 Aplicações*

A nanotecnologia é atualmente uma ferramenta que pode ser bastante útil na quimioterapia experimental. Como certos fármacos possuem características citotóxicas, o uso de carreadores auxiliaria, portanto, na redução dos seus efeitos colaterais. Outra possibilidade é o uso de captação sítio-específica para as células lesadas, sua ação seria direcionada somente às células alvos (lesadas).

Santos *et al.* (2006) observaram que a encapsulação do ácido úsnico reduziu consideravelmente sua hepatotoxicidade. Outros resultados mostraram que dactonomicina D, metotrexato e vinblastina quando encapsulados em nanoparticulas de polialquilcianoacrilato obtiveram efeitos bastante promissores (COUVRER *et al.,* 1980).

Alguns pesquisadores encapsularam inibidores da biossintese do esterol em nanoesferas de poli-lactico-poli-etileno-glicol para testar seu efeito na doença de Chagas e observaram que essas formulações foram efetivas em pequenas doses (MOLINA, 2001). Já González-Mártin *et al.* (2000) encapsularam alopurinol em nanoparticulas de polycianocrilato e verificaram que esta associação com o fármaco revelou uma significante atividade tripanossomicida.

Estudando o mecanismo antitumoral de nanoparticulas de quitosana no carcinoma hepatocelular em testes *in vitro* e *in vivo*, foi observado que estas nanoparticulas exibiram uma atividade antitumoral tempo e dose dependentes em células BEL7402 do hepatocarcinoma humano, mostrando que estas nanoparticulas podem se tornar um potente agente antitumoral no tratamento deste carcinoma (QI *et al.*, 2006).

No entanto, visto que certas substâncias, como os metabólitos liquêncicos, possuem baixa solubilidade em água e alta toxicidade, estudos com encapsulamento dessas substâncias em carreadores de fármacos são necessários, pois podem potencializar seus efeitos.

## 1.2 Líquens e seus metabólitos

### *2.2.1 -Generalidades*

Os liquens são seres formados pela associação simbiótica de um fungo (micobionte) e uma ou mais algas (fotobionte), definição mais comum. No entanto, acredita-se atualmente que são estruturas onde os fungos cultivam algas entre as hifas (XAVIER-FILHO *et al.,* 2006). Existem algumas espécies que estão presentes em rochas, solos e troncos de árvores (MULLER, 2001; NASH III, 1996). São encontrados nos mais diversos habitats, persistindo nas circunstâncias mais extremas, do nível do mar até as montanhas mais altas, desde que estes proporcionem condições necessárias e favoráveis ao seu aparecimento e desenvolvimento (HALE-Jr, 1983; NASH III, 1996, PEREIRA, 1998).

Sob o aspecto morfológico, a estrutura do líquen é formada pelo talo, onde o fotobionte e o micobionte se posicionam em camadas sucessivas. Neste talo, observa-se o córtex superior que é composto por hifas entrelaçadas para proteger a camada das algas. Na parte central, observa-se uma camada frouxa de hifas que é a medula e em seguida outro feixe de hifas, o córtex inferior (Figura 3). A associação formada pelos liquens (alga-fungo) fornece mutuamente proteção e substâncias vitais, satisfazendo suas necessidades e fazendo com que o fungo e a alga possam viver em harmonia (XAVIER-FILHO *et al.,* 2006). O fungo protege a alga da dessecação, através da re-hidratação do talo, após longos períodos de ressecamento (NASH III, 1996) e da intensa luminosidade (HONDA & VILEGAS, 1999) através dos ácidos liquênicos pigmentados. Por outro lado, as algas fornecem vitaminas e substâncias necessárias ao desenvolvimento do fungo (HALE-JR, 1983; NASH III, 1996).

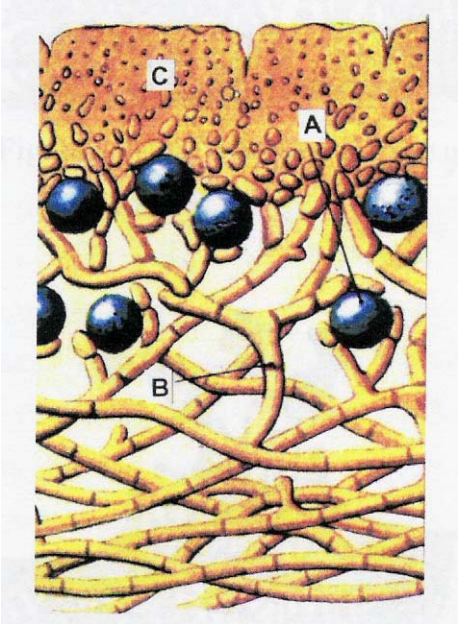


Figura 3. Representação esquemática do talo liquênico: (A) fotobionte; (B) medula; (C) córtex superior.

FONTE: Vasconcelos, 2005.

Metabólitos liquênicos são substâncias extracelulares resultantes do metabolismo secundário. São hidrofóbicos e, para serem extraídos, utilizam-se solventes orgânicos. Normalmente são encontrados nas paredes das hifas (HALE-Jr., 1983). Sua concentração varia de 0,1 a 10% em relação ao peso seco do seu talo. Esses metabólitos

são oriundos das vias: 1- acetato polimalonato, onde são sintetizados as quinonas, depsídeos, depsidonas e ácidos graxos; 2- ácido mevalônico onde são formados os terpenóides e esteróides e 3- ácido chiquímico onde são gerados os derivados do ácido pulvínico (HONDA & VIEGAS, 1998). Já os produtos do metabolismo primário são substâncias hidrossolúveis como polissacarídeos, aminoácidos, proteínas e vitaminas sendo encontrados tanto na parede celular quanto no citoplasma de algas e fungos, podendo ser extraídos com água quente (HALE -JR, 1983).

Encontram-se no Brasil diversas espécies de liquens, dentre elas, *Cladonia substellata, Cladia aggregata e Cladonia salzmannii* que têm como principais compostos o ácido úsnico a primeira; ácido úsnico e barbático a segunda e ácido barbático terceira espécie.

### 2.2.2 *Ácido Barbático*

A *C. salzmannii* distribui-se nos diversos Estados brasileiros, como Bahia, Sergipe, Pernambuco, Minas Gerais e Paraíba. A principal substância encontrada nesta espécie é o ácido barbático ($C_{16}H_{20}O_7$), sendo ocasionalmente encontrados os ácidos taminólico, D-taminólico e o ácido 4-O-dimetilbarbático (AHTI *et al*., 1993).

O ácido barbático (figura 4) é um depsídeo formado por dois anéis aromáticos interligados entre si por uma ligação éster. Em um grupamento do anel, apresenta um grupo carboxílico (PEREIRA, 1998). Seus cristais possuem cor amarelo-pálido e dissolvem-se facilmente em éter, etanol, acetona e cloforfórmio (ASAHINA & SHIBATA, 1954; HUNECK & YOSHIMURA, 1996).

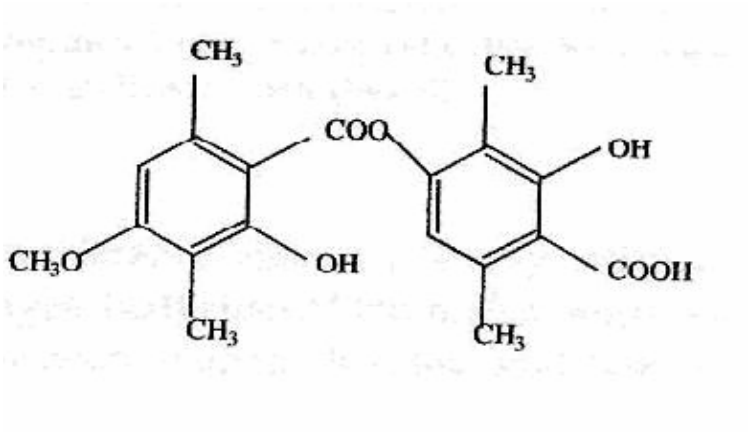


Figura 4. Estrutura química do ácido barbático segundo Huneck & Yoshimura (1996)

### *2.2.3 Atividades Biológicas*

Dentre os efeitos biológicos das substâncias liquênicas, destacam-se os efeitos antiviral, antibiótico, antifúngico (PEREIRA *et al*., 1994; 1996; PERRY *et al.,* 1999; PIOVANO, 2002,) antineoplásico (CULBERSON, 1970; PEREIRA *et al.,* 1991; 1994; NASCIMENTO *et al*., 1994) inibidor do crescimento de plantas superiores e inibidor enzimático (HUNECK & YOSHIMURA, 1996).

Extratos orgânicos de espécies de liquens são ativos contra bactérias álcool-ácido resistentes, como o *Mycobacterium tuberculosis* (CAPRIOTTI, 1961). Estudos com o ácido barbático mostraram excelentes resultados contra o *B. subtilis* (PERRY *et al.*, 1999). Testes com os ácidos úsnico, lobárico, protoliquesterínico e salazínico mostraram que estes foram ativos contra o *M. aurum*, quando comparados a drogas antibacterianas como, rifampicina, estrepetomicina e isoniazida (INGOLFSDOTTIR *et al.*, 1998).

Os mecanismos da ação antibiótica dos metabólitos sugerem que estes modificam a estrutura da proteína, o que resulta em alteração no metabolismo celular e alterações irreversíveis na célula podendo conduzí-la à morte (VICENTE *et al.,* 1995).

Estudos verificaram a eficácia do ácido úsnico contra a leishmaniose cutânea utilizado intralesionalmente (FOURNET *et al.,* 1997). Pesquisas demonstraram que esta substância extraída de *C.substelatta* é ativa contra as formas evolutivas do *T. cruzi*, mostrando ser assim um possível agente quimioterápico contra a doença de Chagas (CARVALHO *et al.*, 2005). SANTOS *et al* (2006) aplicaram nanocápulas de PLGA contendo ácido úsnico em ratos com sarcoma 180 e verificaram que estas aumentaram a atividade antitumoral e reduziram a hepatotoxicidade desta droga, sugerindo assim que a nanoencapsulação pode ser um meio de introduzir o ácido úsnico na quimioterapia. De certa forma, é interessante estudar a ação de metabólitos liquênicos na quimioterapia de doenças parasitárias como leishmaniose e doença de Chagas.

## **2.3 *Trypanosoma cruzi***

### *2.3.1 Doença de Chagas*

A doença de Chagas é uma enfermidade infecciosa e parasitária causada pelo protozoário *Trypanosoma cruzi* (*T. cruzi*). Esta doença foi descoberta em 1909 pelo pesquisador brasileiro Dr. Carlos Justiniano Ribeiro das Chagas, quando este realizava uma campanha contra a malária que atingia operários que trabalhavam na construção de um trecho da Estrada de Ferro Central do Brasil, na região norte do Estado de Minas Gerais.

Segundo a Organização Mundial da Saúde, existem cerca de 18 milhões de pessoas portadoras da doença na América Latina e mais de cinco milhões de brasileiros infectados, sendo, portanto um problema mundial de saúde. A doença de Chagas causa altas taxas de morbidade e mortalidade no mundo todo. Esta enfermidade é um exemplo típico de uma injúria orgânica resultante das alterações produzidas pelo ser humano ao meio ambiente, das distorções econômicas e dos problemas sociais (VINHAES and DIAS, 2000).

A doença de Chagas caracteriza-se por três formas: fase aguda, fase indeterminada e fase crônica. A fase aguda inicia-se logo após a infecção com um período de incubação de sete dias. Podendo ser assintomática ou haver sintomas como febre, mal-estar, anemia, anorexia, hepatomegalia e esplenomegalia, esses dois últimos mais comuns nas crianças. Na fase indeterminada, o individuo pode permanecer um bom tempo como um reservatório de parasito. No entanto, aproximadamente um terço das pessoas contaminadas nesta fase desenvolverá a doença de Chagas crônica. Os portadores da doença na fase crônica podem apresentar mais duas formas que são a cardíaca e a digestiva de acordo com as características acometidas (BRENER *et al.,* 2000).

### *2.3.2 Morfologia e ciclo de vida do T. cruzi*

O *T. cruzi* é um protozoário hemoflagelado pertencente à família Trypanomastidae da ordem kinetoplastida. Tem um núcleo oval, um flagelo e um cinetoplasto que é uma mitocôndria especializada rica em DNA. Possui três formas distintas em seu ciclo de vida: a tripomastigota, amastigota e a epimastigota, que se diferenciam morfologicamente pela posição de emergência do flagelo e pela posição do cinetoplasto em relação ao núcleo (figura 5) (HOARE & WALLACE 1996).

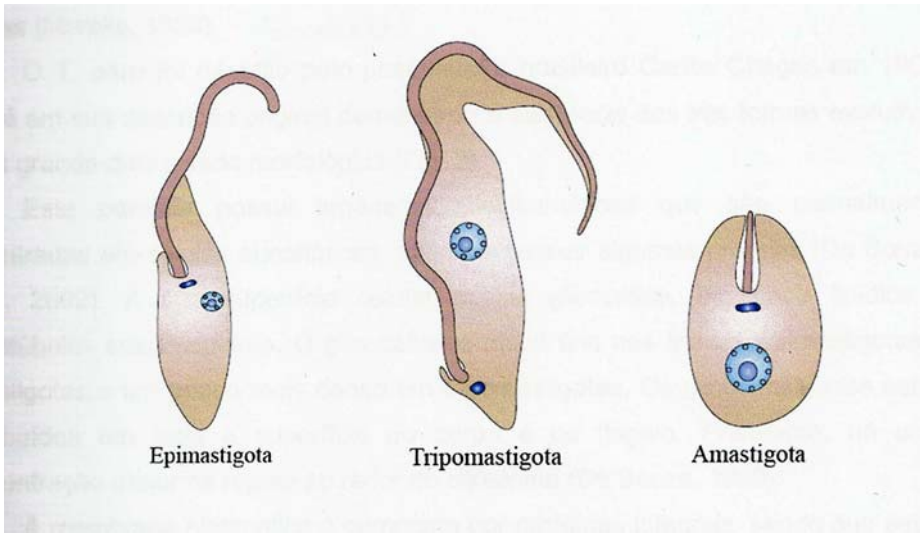


Figura 5. Representação esquemática das três formas evolutivas do *Trypanosoma cruzi*. (Adaptado de DoCampo *et al.*, 2005).

A forma tripomastigota é a forma infectante, encontra-se no intestino do triatomíneo assim como na corrente sanguínea. Tem um corpo fino e seu cinetoplasto é volumoso em forma de cesta. Este se encontra na parte posterior, junto com a emergência do flagelo. A amastigota é encontrada nas células dos hospedeiros mamíferos e corresponde ao estágio multiplicativo. Apresenta forma arredondada e seu cinetoplasto é em forma de bastão. A forma epimastigota é encontrada no tubo digestivo do inseto vetor. Possui um corpo alongado e fusiforme. Seu cinetoplato é em forma de bastão e se encontra na posição anterior. A emergência do flagelo se dá lateralmente ao corpo celular (HOARE & WALLACE, 1996).

Os tripanossomatídeos possuem organelas citoplasmáticas comuns às células eucarióticas como o complexo de golgi, o retículo endoplasmático e o núcleo. Além de organelas que lhes são peculiares como cinetoplasto, glicossomos, acidocalcissoma e reservossomos, estes últimos encontrados principalmente na forma epimastigota do *T.cruzi* (figura 6) (DE SOUZA, 2002).

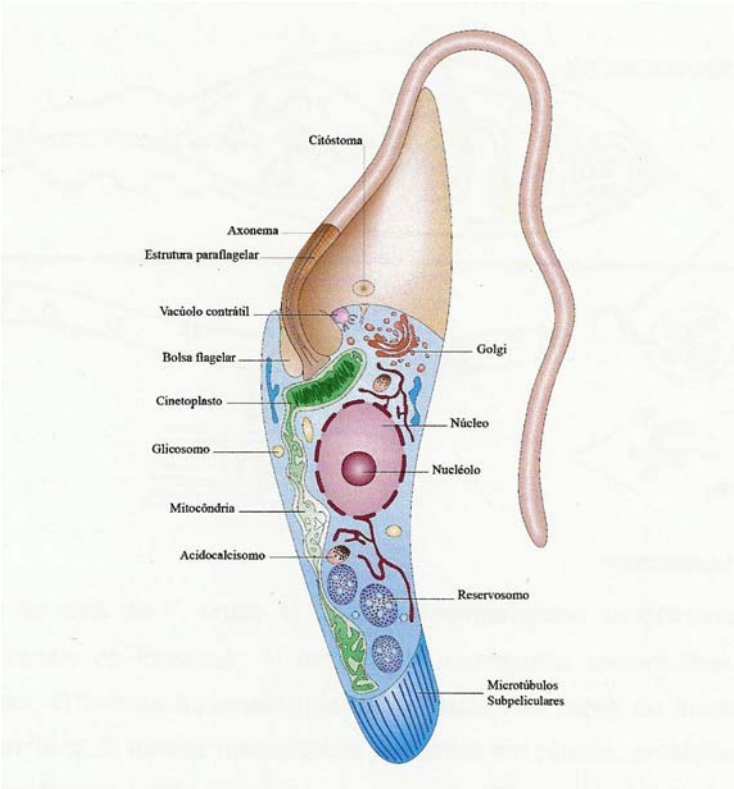


Figura 6. Esquema mostrando as organelas citoplasmáticas em uma forma epimastigota de *Trypanosoma cruzi*. (Adaptado de DoCampo *et al.*, 2005).

A membrana plasmática é composta por proteínas integrais, sendo que estas estão presentes em maior número na face interna da membrana de epimastigotas do que em amastigotas e tripomastigotas. Os lipídeos e carboidratos estão dispostos na superfície externa da membrana (DE SOUZA, 1999).

Os glicossomos são peroxossomos que possuem enzimas envolvidas na via glicolítica, são responsáveis pela glicólise neste organismo (DOCAMPO & MORENO, 2001). Os alcidocalcissomas são organelas que possuem em seu interior um material eletro-denso, são responsáveis pela homeostase dos íons cálcio e fósforo nos tripanossomatídeos. Os reservossomos são pequenas vesículas que servem de armazenamento das macromoléculas ingeridas pela célula. Estão situados na parte posterior da forma epimastigota e seu diâmetro é cerca de 0,4-0,5 μm (FIGUEIREDO & SOARES, 2000).

O ciclo de vida do *T. cruzi* inicia-se quando o triatomíneo ao sugar o animal parasitado, se infecta com as formas tripomastigotas, que sofrem modificações ao longo do tubo digestivo. Após poucas horas do repasto, no estômago, os tripomastigotas ingeridos sofrem modificações e se transformam em epimastigotas e amastigotas.

Posteriormente, essas últimas migram para o reto e, por interações hidrofóbicas do flagelo com a parede da cutícula do intestino do inseto, aderem-se nessa região e se transformam em tripomastigotas metacíclicas infectantes que são liberadas nas fezes do inseto. Através da pele com solução de continuidade ou das mucosas, os tripomastigotas metacíclicas penetram no interior de qualquer célula, exceto eosinófilos e neutrófilos. Dentro das células, as tripomastigotas sofrem um processo de regressão, transformando-se em amastigotas, que se multiplicam por divisões binárias sucessivas, formando pseudocistos. Após cinco dias aproximadamente, as formas amastigotas evoluem para a forma tripomastigota sanguínea. Devido ao intenso movimento flagelar, ocorre ruptura da célula hospedeira liberando grande quantidade de tripomastigotas e menor número de amastigotas. As três formas são capazes de infectar outras células do hospedeiro vertebrado, sendo capturado pelo inseto, durante o repasto sanguíneo, finalizando o ciclo (figura 7) (TYLER & ENGMAN, 2001; DE SOUZA, 2002).

Figura 7. Ciclo biológico do *T. cruzi.*

http://www.who.int/tdr/diseases/chagas/lifecycle.htm

*2.3.3 Terapêutica*

Infelizmente, apesar do grande avanço no entendimento da biologia do *Trypanosoma cruzi*, nos estudos das vias bioquímicas e na descoberta de compostos tripanossomicidas, as duas únicas drogas contra este protozoário ainda são as mesmas que foram registradas há mais de 20 anos atrás, nifurtimox e benzonidazol (CROFT *et al.,* 2005). No entanto, o nifurtimox foi retirado do mercado farmacêutico (DOCAMPO & URBINA, 2003; FERREIRA, 1990).

O nifurtimox desestabilizava os radicais nitroânions que se tornam reativos e produzem alta toxicidade. Já o Benzonidazol atua por um mecanismo diferente, que

envolve modificações covalentes de macromoléculas, como o DNA por nitroredução dos intermediários, levando a perda da capacidade proliferativa do parasita (DOCAMPO & URBINA, 2003). Entretanto, ambos componentes podem reduzir os efeitos da fase aguda. Já na fase crônica é um problema a ser discutido por não terem eficácia (ESTANI *et al.,* 1998). Alguns autores demonstraram que interleucinas e o interferon estão associados ao sistema imune e é promissor na cura parasitológica induzida pelas duas drogas (ROMANHA, 2002). Essas drogas apresentam alguns efeitos colaterais como conseqüência dos danos da oxidoredução nos tecidos do hospedeiro. Esses efeitos colaterais incluem anorexia, vômitos, polineuropatia periférica, leucopenias e dermatopatias alérgicas (KIRCHOFF, 1999).

Muitos pesquisadores estão estudando novos quimioterápicos para combater esta doença de modo mais eficiente e menos tóxica para o homem. Estudos verificaram a redução da parasitemia em camundongos em derivados de 8-aminoquinolines, sendo estes tão eficientes quanto o Nifurtimox (KINNAMON *et al.,* 1997) Pesquisas mostraram cura e sobrevivência entre 80 a 100% dos camundongos na fase aguda e crônica da doença através do uso via oral do triazole TAK-187 (URBINA *et al.,* 2003). URBINA (2003) e colabaradores demonstraram também que o ravuconazol tem um efeito potente e específico contra o *T. cruzi* na forma aguda da doença, porém esse efeito não foi satisfatório na fase crônica.

Tratamento de ratos infectados por *T. cruzi* administrando extratos de própolis oralmente parece interferir na propriedade básica do sistema imunológico dessas células infectadas, levando a uma diminuição dos níveis de parasitemia sem efeito tóxico renal até em doses altas administradas (DANTAS *et al*., 2005).

Existem uma série de compostos com promissora atividade tripanossomicida dentre eles estão, os análogos dos lisofosfolipídeos como o miltefosine, que tem destacada atividade oral contra leishmanias e atividade *in vivo* nas infecções experimentais de *T. cruzi*; e inibidores do fenil e N-miristoil transferase, como potenciais agentes tripanossomicidas (GELB, 2003). Pesquisas utilizando alternativas terapêuticas, como plantas contra protozoários, mostraram que estas podem ser ativas contra leishmania e malária (WENIGER *et al.*, 2001).

Derivados de triazol foram capazes de induzir cura parasitológica total em modelos murinos nas fases crônica e aguda da doença. Além do mais, tais compostos foram capazes de erradicar cepas resistentes ao nitroimidazol e ao nitrofurano presentes em ratos infectados, até se o hospedeiro estivesse imunossuprimido (URBINA, 2002).

Recentemente, outros triazólicos tais como ravuconazol, também têm mostrado atividade tripanossomicida *in vivo* e *in vitro* (URBINA *et al.,* 2003).

A via de biossintese do ergosterol é um excelente alvo para a terapia contra o *T. cruzi,* pois este requer esteróis específicos para a viabilidade e proliferação das suas células em todos os estágios do seu ciclo de vida. Desta forma, o *T. cruzi* é extremamente susceptível aos inibidores desta via. Pesquisadores observaram que inibidores do ergosterol apresentaram um bom efeito tripanosomicida contra cepas do *T. cruzi*. Demonstraram também que o gene da enzima C14-α demetilase é expresso no parasito tanto nos estágios do ciclo de vida no mamífero, quanto no inseto, sendo potencialmente um alvo metabólico muito importante (Buckner *et al*., 2003).

Estudos utilizando camundongos infectados por *T. cruzi* mostraram que Citocinas e linfócitos influenciam o desenvolvimento da fase aguda da doença de Chagas experimental,

camundongos deficientes em IFN-γ apresentaram uma parasitemia ascendente que atinge um nível médio 7 vezes maior do que o pico da curva de camundongos normais e camundongos deficientes em qualquer um dos efetores estudados infectados com o *T. cruzi* morrem precocemente quando comparados aos camundongos normais (FERRAZ, 2005).

Assim como na quimioterapia de outras doenças, na doença de Chagas deve-se priorizar o mínimo de efeito colateral e o máximo de eficácia da droga. Diante de todo o conhecimento da biologia e bioquímica do *T. cruzi* é necessário o desenvolvimento de compostos eficazes que direcionem sua ação, com isso a tecnologia farmacêutica através do uso de drogas sítio-específicos é uma boa alternativa (COURA & DE CASTRO, 2002). Excelentes resultados foram obtidos com formulações de anfotericina no tratamento clinico da leishmaniose (SUNDAR *et al.,* 1998). A incorporação de inibidores da biossíntese do ergosterol em nanoesferas de ácido polilático-polietilenoglicol além de aumentar a biodisponibilidade destes compostos pouco solúveis, aumenta também a cura em testes experimentais com animais (MOLINA *et al.*, 2001).

## 3. OBJETIVOS

### *3.1 Geral:*

- Avaliar o potencial de nanocápsulas de PLGA contendo ácido barbático como droga tripanosomicida

### *3.2 Específicos:*

- Obter e caracterizar nanocápsulas de PLGA contendo ácido barbático, através de análises físico-quimicas;
- Avaliar os efeitos do ácido barbático livre e encapsulado sobre o crescimento e proliferação de formas epimastigota de *T. cruzi*, através da contagem de células;
- Avaliar os efeitos do ácido barbático livre e encapsulado sobre a ultraestrutura de formas epimastigotas de *T. cruzi* através de microscopia eletrônica de transmissão.

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## 5. ARTIGO A SER SUBMETIDO À PUBLICAÇÃO

# *In vitro* Activity of Free and Nanoencapsulated Barbatic Acid against *Trypanosoma cruzi*

**ao**

**“ International Journal of Pharmaceutics”**

# *In vitro* Activity of Free and Nanoencapsulated Barbatic Acid against *Trypanosoma cruzi*

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**Abstract**

The aim of this study was to evaluate the effects of barbatic acid (BARB) on the viability, growth and ultrastructure of *Trypanosoma cruzi* epimastigote forms, the ethiologic agent of Chagas' disease. In order to improve the BARB bioavailability this compound was incorporated into D,L-(lactide-glycolide) copolymer (PLGA) nanocapsules (BARB-NC). The cell growth and viability was evaluated by cell counting using Neubauer chamber for 24, 48 and 72 hours. The parasites were incubated at different BARB or BARB-NC concentrations for 72 hours estimated after 24 hours of cultivation. Parasites grown in drug free medium were used as a control. The incubation of parasites with unloaded nanocapsules resulted in no inhibitory activity in comparasion with control cells, but slight ultrastructural changes were verified. Treatment of parasites with both BARB and BARB-NC resulted in a dose-dependent growth inhibition. BARB caused an inhibition of only 32% of parasite proliferation at 62.5 μg/ml at 24 hours. However, the $IC_{50}$ value of BARB-NC at 24 hours was 24.66 μg/ml. The incorporation of BARB into nanocapsules enhanced therefore its trypanocidal activity. Nevertheless the treatment of epimastigotes with both BARB and BARB-NC caused drastic morphological changes with a number of affected cells and severe ultra-structural alterations considerably higher in BARB-NC treated parasites.

**Keywords:** barbatic acid, nanocapsules, *Trypanossoma cruzi*

## INTRODUCTION

Chagas' disease is endemic in Latin America affecting about 18 million people, (WHO, 2002). This disease is a substantial cause of morbidity and mortality in all endemic countries being responsible for large social economical losses (Do Campo and Schmunis, 1997). The etiologic agent of Chagas' disease is the haemoflagellate *Trypanosoma cruzi*, which is transmitted to humans by triatomine insect vectors, blood transfusion and congenital routes. There are no vaccines available to the prophylaxy of Chagas' disease and its chemotherapy remains rather unsatisfactory. The first-choice treatment still relies on the administration of nifurtimox and benznidazole drugs. However, the commercial production of nifurtimox has been discontinued in Brazil since the 1980s and more recently in further Latina America countries (Docampo & Urbina, 2003). Both antitrypanosomial drugs have also a significant toxicity, severe side effects and limited efficacy in the chronic phase of such a disease. Moreover, some *Trypanosoma cruzi* strains present resistance to such drugs (Filardi & Brenner, 1987). In this challenging scenario, there is a need for novel chemotherapic approaches for the treatment of this serious illness (Kierszenbaum, 2005).

Lichens –symbiontic organisms of fungi and algae– can synthesize numerous metabolites, which comprise aliphatic, cycloaliphatic, aromatic, and terpenic compounds. The effects of lichen compounds have long been studied (Vartia, 1973). Many metabolites have a manifold biological activity including antiviral, antibiotic, antitumor, allergenic, plant growth inhibition, and enzyme inhibitory properties (Lauterwein *et al*., 1995; Ingolfsdottir *et al*., 1998; Ingolfsdottir, 2002, Yanmamoto *et al.*, 1995; Perry *et al.*, 1999, Huneck & Yoshima 1996, Cocchietto *et al*., 2002). Concerning to pathogenic protozoa, studies have shown that some lichen compounds were effective against relevant parasites. De Carvalho *et al.* (2005) showed that usnic acid from *Cladonia substelatta* is effective against all *Trypanosoma cruzi* evolutive forms. In addition, intralesional administration of usnic acid in Leishmania infected BALB/c mice resulted in a significant reduction in cutaneous lesions (Fournet *et al.*, 1997).

The advent of nanotechnology with polymeric carriers could be a promissory tool for delivering the required doses for prolonged time periods by a single shot, without causing toxicity (Reis *et al*. 2006). Furthermore, these nanosystems can protect drugs from degradation on biological fluids and may be able to improve the efficacy of the

active agent (Barrat, 2000; Whelan, 2001). Additional advantages of these systems are suitable stability, controlled release, improved absorption and the expected pharmacodynamic acitivity (Santos-Magalhães *et al*., 2000). The nanoencapsulation of lichen compounds could improve their effects, since these substances have high toxicity (Han *et al*. 2004) and hydrophobic properties that can limited their applications.

The barbatic acid ($C_{19}H_{20}O_7$) (Figure 1) is one of the lichen-derived depside that has shown strong inhibitory activity against tumour promoter-induced Epstein-Barr virus (Yamamoto *et al*., 1995). It has a melting point of 187ºC and it is extremely hydrophobic (Asahina & Shibata, 1954). This compound has also been showed to be promissory as an herbicide by inhibiting the electron transport in the photosystem II (Endo *et al*., 1998). Diffratic acid is a congener of barbatic acid, which exhibit antiviral, antitumor, analgesic and antipyretic activity (Huneck & Yoshimura, 1996). On the other hand, the barbatic acid data reported in literature is somewhat lacked.

In the present work we developed nanocapsules containing barbatic acid purified from *Cladonia salzmannii* in order to verify its antiproliferative activity on epimastigote forms of *Trypanosoma cruzi*. In this way, the effects of both free and encapsulated barbatic acid on the growth, proliferation and ultrastructural morphology of these parasites were investigated.

## MATERIALS AND METHODS

### Chemicals and drug

Poly (d,l-lactic-co-glycolic acid) (PLGA 50:50) was purchased from Birmingham Polymers (Alabama, USA); soybean phosphatidylcholine (Epikuron® 200) was obtained from Lucas Meyer (Germany); poloxamer 188 was generously supplied by ICI (France); soybean oil was purchased from Sigma-Aldrich (St. Louis, USA); monobasic potassium phosphate, sodium hydroxide, methanol, dimethyl sulfoxide (DMSO) and analytical grade solvents were obtained from Merck (Darmstadt, Germany). Barbatic acid was obtained from *Cladonia salzmannii* according previously reported (Pereira *et al*., 1994). The resin used in transmission electron microscopy was the Polybed 812 (Polyscience, Warrington, PA, USA).

### Preparation of barbatic acid-loaded nanocapsules

The barbatic acid-loaded nanocapsules (BARB-NC) were prepared with d,l-(lactic-glycolic acid) copolymer (PLGA) using the solvent displacement method (Fessi *et al.,* 1989). Briefly, the polymer (150 mg), the soybean oil (100 mg), the soya phospholipid (150 mg) and the barbatic acid were each dissolved in acetone and then mixed under magnetic stirring at 150 rpm for 10 min. The aqueous phase consisted of the polaxamer®188 (150 mg) dissolved in 50 ml of 0.2 M phosphate buffer solution, pH 7.4. The organic solution (27 ml) was then gradually poured into the aqueous phase under magnetic stirring at 150 rpm for 30 min. Nanocapsules were spontaneously produced and the solvent was removed under reduced pressure at 40ºC. The colloidal suspension of nanocapsules was then concentrated to 10 ml final volume by removing the water at similar conditions. Different batches of nanocapsules were formulated with barbatic acid at 0.5, 0.7 and 1 mg/ml concentrations. Unloaded nanocapsules were also prepared using the above described conditions.

In order to simulate sterilization, transport and storage, the nanocapsules were submitted to both accelerated and long-term stability testing. An aliquot (1 ml) of a suspension of babartic acid-loaded nanocapsules was submitted to centrifugation (6,000 rpm for 1 hour) and mechanical agitation (140 strockes/min for 48 hours at 25°C). Long-term stability testing was conducted to evaluate formulation durability during 30

days. The macroscopic and microscopic appearances of nanocapsules were observed and pH was measured after each test.

**Parasites**

*T. cruzi* epimastigote forms (Dm28c clone) were maintained in liver-infusion medium (LIT) supplemented with 10% fetal bovine serum at 28ºC (Camargo, 1964) and harvested at the exponential phase of growth at 24 to 72h.

***In vitro* effect of barbatic acid and barbatic acid-loaded nanocapsules on *Trypanosoma cruzi***

Epimastigote forms were ressuspended in LIT medium at a density of 7 x $10^6$ cells/ml and seeded in 24-well plates. 500 µl of this culture suspension was then added to the same volume of free or encapsulated barbatic acid, previously prepared in LIT medium at the desired concentration (3.9 - 62.5 µg/ml). Incubations were performed for 72 hours and viable parasite was determined by cell counting using Neubauer chamber after 24, 48 and 72 hours of cultivation. The drug concentration require to inhibit cell growth in 50% ($IC_{50}$) was estimated by the logarithm adjustment of the percentage of parasite inhibition versus drug concentration curve. The results were expressed as mean ± standard deviation of three independent countings. The motility and morphology of the parasites was monitored by light microscopy.

**Statistical analysis**

The statistical analysis were performed using Mann-Whitney and Kruskall-Wallis tests, assuming a 5%confidence level ($p<0.05$). The statistical package software was the 8.0 SPSS version.

**Transmission electron microscopy**

Epimatigotes were incubated with BARB (83.45 µg/ml) and BARB-NC (31.16 µg/ml) for 24 hours. Then the parasites were collected by centrifugation at 3000 *g*, washed in 0.1 M phosphate buffer at pH 7.2 and fixed for 2 hours with 2.5% glutaraldeyde and 4% paraformaldeyde (1:1) in 0.1 M phosphate buffer solution. The cells were then washed with the buffer solution and post-fixed for 15 min with 0.1 M

cacodylate buffer at pH 7.2 containing 0.1% osmium tetroxide and 0.8% potassium ferricyanide in 5 mM calcium chlorure. After rinsing with buffer solution, the cells were dehydrated with graded acetone, and embedded in Polybed 812 resin. Ultrathin sections were stained with 5% uralnyl acetate and citrate and observed in a transmission electron microscope (Zeiss EM109). Parasites incubated with free drug medium or unloaded nanocapsules were both used as controls.

## RESULTS

### Stability of barbatic acid-loaded nanocapsules

Results showed that the most stable formulation of barbatic acid-loaded nanocapsules was achieved at a drug concentration of 0.5 mg/ml. All formulations at concentrations beyond this value exhibited aggregates and drug crystal precipitation. The barbatic acid-loaded nanocapsule suspensions were submitted to both accelerated and long-term stability testing. As far as accelerated stability evaluation is concerned, it was showed that just one formulation (0.5 mg/ml) presented stability on transport simulation testing by mechanical agitation (Table 1). The BARB-NC (0.5 mg/ml) formulation maintained its initial macroscopic appearance when submitted to mechanical stress resistance testing. This formulation remained stabilized without any noticeable physicochemical changes after submitted to centrifugation. The macroscopic examination showed a milk opalescent appearance with bluish reflection, which was maintained for 15 days. The long-term stability of 0.5 mg/ml of barbatic acid-loaded nanocápsulas was evaluated and it was observed the presence of microcapsules (Table 2). The presence of few microcapsule was observed but no oil droplets or drug crystals were verified by light microscopy at all tested concentrations.

**Antiproliferative ffects of free and encapsulated barbatic acid on epimastigotes**

The treatment of epimastigote forms of *T. cruzi* with free barbatic acid at different concentrations resulted in a dose-dependent growth inhibition only after 48 hours of incubation (Fig. 2A), whereas treatment with barbatic acid-loaded nanocapsules showed significant inhibitory effect as soon as 24 hours of incubation (Fig. 2B). Paradoxically no stastitical differences were found between both treatments after 48 and 72 hours of incubation for both free and encapsulated barbatic acid. No difference in the parasite proliferation was found when the parasites were incubated in presence of unloaded nanocapsules in comparison to the control for any time of incubation.

The percentage of parasite growth inhibition is shown in Fig 3. It can be seen that the effect of free and encapsulated barbatic acid against *T. cruzi* epimastigotes was dose-dependent and it was improved by nanoecapsulation. In fact, a remarkable improvement on the inhibition of parasite proliferation was achieved with barbatic acid-loaded nanocapsules for concentrations higher than 15.62 μg/ml (42 %). Free barbatic acid at this concentration inhibts merely 16 % of the parasite growth. The $IC_{50}$ of the barbatic acid-loaded nanocapsules, estimated by linear fitting of parasite growth inhibition (%) versus log drug concentration curve, was about 25 μg/ml at 24 h. However, the $IC_{50}$ of the free barbatic acid could not be determined since a maximum inhibition of only 32% was achieved at 62.5 μg/ml.

**Ultra structural effects of free and encapsulated barbatic acid on epimastigotes**

Transmission electron microscopy analysis of epimastigotes showed that the treatment with free and nanoencapsulated barbatic acid caused several changes in cell morphology (Fig 4). Untreated parasites presented a centrally located nucleus, a compact condensation of mitochondrial kinetoplast and reservosomes, the latter corresponding to the pre-lysosomal compartment where small lipid droplets and endocytosed proteins are accumulated (Fig. 4A). A single Golgi complex was always observed close to the flagelar pocket and the kinetoplast (Fig. 4B). However, the treatment of epimastigotes with free barbatic acid (Figs. 4C and D) or barbatic acid-loaded nanocapsules (Fig. 5B-D) caused drastic ultrastructural morphological changes.

These alterations included cellular disorganization, extraction of cytoplasm content and rupture of organelles as the reservosomes and the Golgi complex. The mitochondrial kinetoplast was rarely affected by both treatments. Although the ultrastructural effects in epimastigotes after both treatments were similar, the number of affected cells and the severity of morphological changes were considerably higher in barbatic acid-loaded nanocapsules treated parasites. The reservosomes presented the major morphological changes in BARB-NC treated epimastigotes, with the loose of their content and the appearance of membranous profiles inside it (Figs 5C). Vacuoles contained membranous profiles and lipid-like inclusions were also commonly seen in cells treated with barbatic acid-loaded nanocapsules (Fig. 5D). Treatment with unloaded nanocapsules caused slight morphological alterations as compared as parasites incubated with free drug medium, which include an increase in the number of lipid inclusions and displacement of some organelles (Fig. 5A).

## DISCUSSION

Although lichen metabolites have shown to have a broad-spectrum of biological activities, the pharmacological application of these compounds have often been overloaded owing to their poor solubility in non-toxic solvents (Kristmundsdóttir et al., 2005**,** Erba *et al*., 1998) as well as their toxicity (Fraveau *et al*., 2002; Han *et al*., 2004). Due to their oily cavity and small size, polymeric nanocapsules might be promising as carriers of insoluble lichens compounds, by providing both sufficient drug solubility and improving their penetration into the cells. In this way, Santos *et al*. (2006) showed that the encapsulation of usnic acid into poly-lactic-glycolic acid (PLGA)-nanocapsules was able to maintain and improve its anti-tumor activity whereas considerably reduced their hepatotoxicity. As the usnic acid, barbatic acid is also a hydrophobic compound that can efficiently be encapsulated into PLGA-nanocapsules.

In order to evaluate the usefulness of barbatic acid-loaded nanocapsules, their *in vitro* effects on growth and proliferation of epimastigote forms of *T. cruzi* were evaluated in comparison with the free drug treatment. A reduction in cell proliferation was found for both treatments with free and nanoencapsulated barbatic acid point to an effect on growth and viability of epimastigote forms, mainly at 15-62.5 μg/ml drug concentrations. However, a remarkable trypanocidal activity of the barbatic acid-loaded

nanocapsules, after 24 hours of incubation, whereas the inhibitory effect of BARB was observed only after 48 hours. The existing difference between these treatments could be explained by the hydrophobic nature of barbatic acid (Müller, 2001) leading to the lower solubility of this compound in the culture medium. In this sense, our results showed that the barbatic acid encapsulation enhance the availability of this lichen compound to the cells, probably by allowing a suitable interaction between the nanoparticles surface and the parasite membrane (Couvreur *et al*., 1984).

In this investigation the unloaded-PLGA nanocapsules showed have no activity on parasite proliferation. Similar results were obtained by Molina *et al*. (2001) with polyethiyleneglycol-polylactide nanospheres, which have no cytotoxic effects of in treated mice. Nevertheless, Gonzalez-Martin (2000) reported that unloaded polycyanoacrylate-nanoparticles presented an intense trypanocidal activity. They suggested that enzymatic degradation of polycyanoacrylate could produce formaldehyde that would contribute to the cellular death. These data together demonstrated that the chemical composition of nanocapsules must be taken into account in cytotoxity assays. Although no trypanocidal activity was observed, the incubation of cells with unloaded-PLGA nanocapsules caused slight ultrastructural changes in the parasites, such as organelles displacement and accumulation of lipid droplets. The incorporation of nanocapsule elements, mainly the oily phase and phospholipids may cause an imbalance of lipids into cell. The accumulation of lipid inclusions in parasites treated with unloaded and drug-charged nanocapsules may be a way found by the parasites to deal with exceeding lipids.

One conjecture for the mechanism of action of barbatic acid is its accumulation in cell membranes, thereby resulting in metabolites. Diffractaic and barbatic acids are non-redox inhibitors of the biosynthesis of leukotrienes in mammalian cells. Leukotrienes are derived from the biotransformation of arachidonic acid through the action of 5-lipoxygenase and play an important role in a variety of human physiopathology (Kumar *et al*., 1999). In *Trypanosoma brucei*, archidonic acid has been shown to be decisive in calcium influx regulation. The inhibition of this pathway can block the calcium influx through the plasma membrane, getting the cell death (Eintracht *et al*., 1998). Thus, another possibility is that barbatic acid act in some step of arachdonic biosynthesis pathway.

## CONCLUSION

In conclusion, our results confirm the encapsulation efficiency of barbatic acid into PLGA-nanocapsules. In addition, the barbatic acid-loaded nanocapsules may improve its activity against *Trypanosoma cruzi* at the first 24 hours of incubation, causing severe ultrastructural modifications in cells. Regardless of additional studies are required to elucidated the ensemble of pathophysiological effects of free and encapsulated barbatic acid, these formulations can be offered as chemotherapy agents for Chagas' disease.

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**FIGURE 1.**

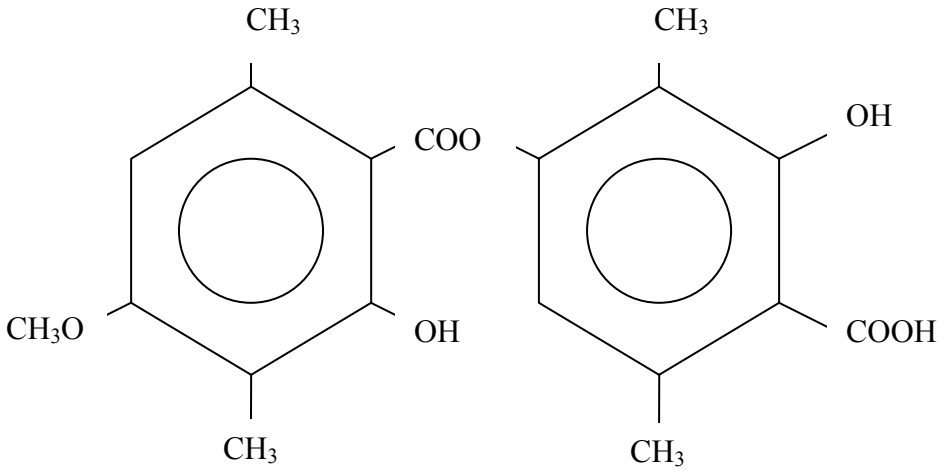


**FIGURE 2 A.**

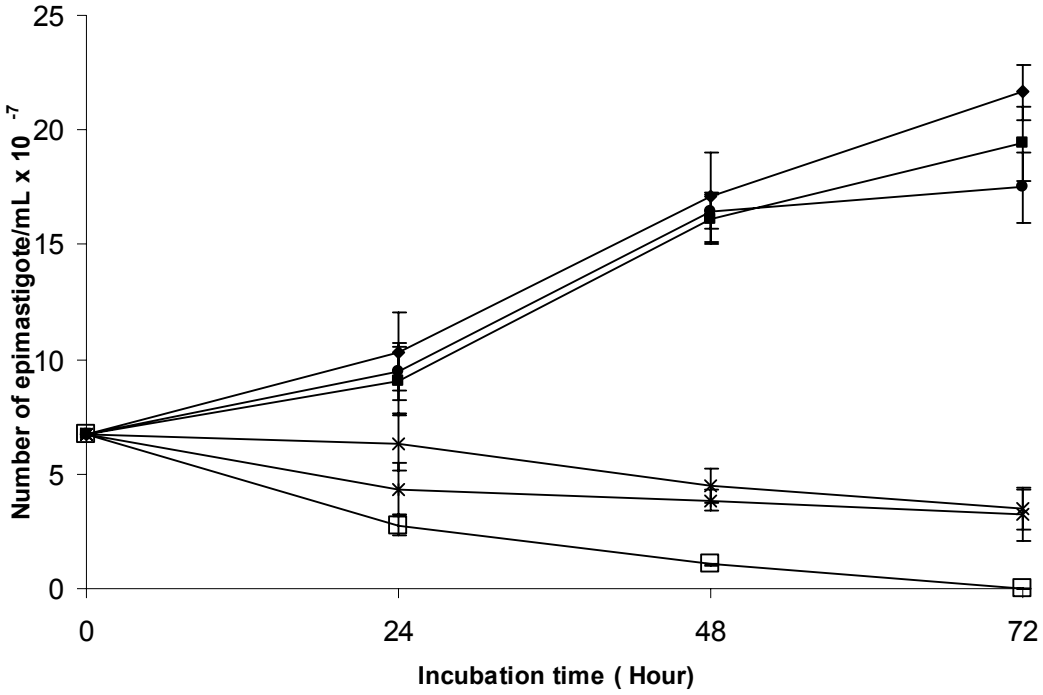



**FIGURE 2 B.**

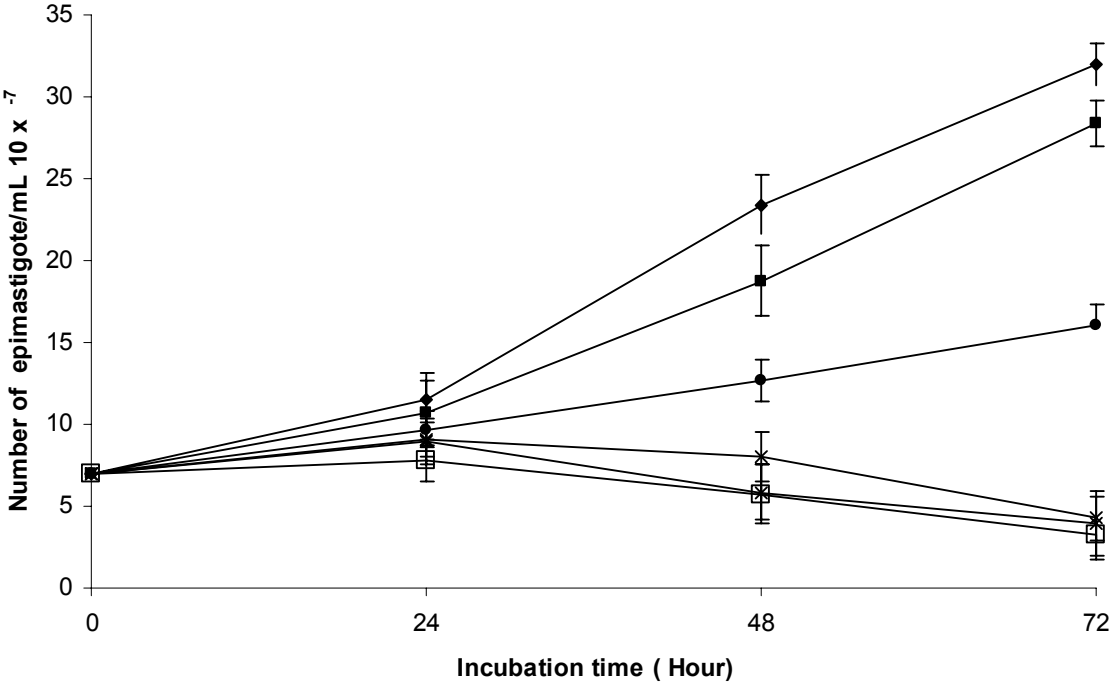


**FIGURE 3.**

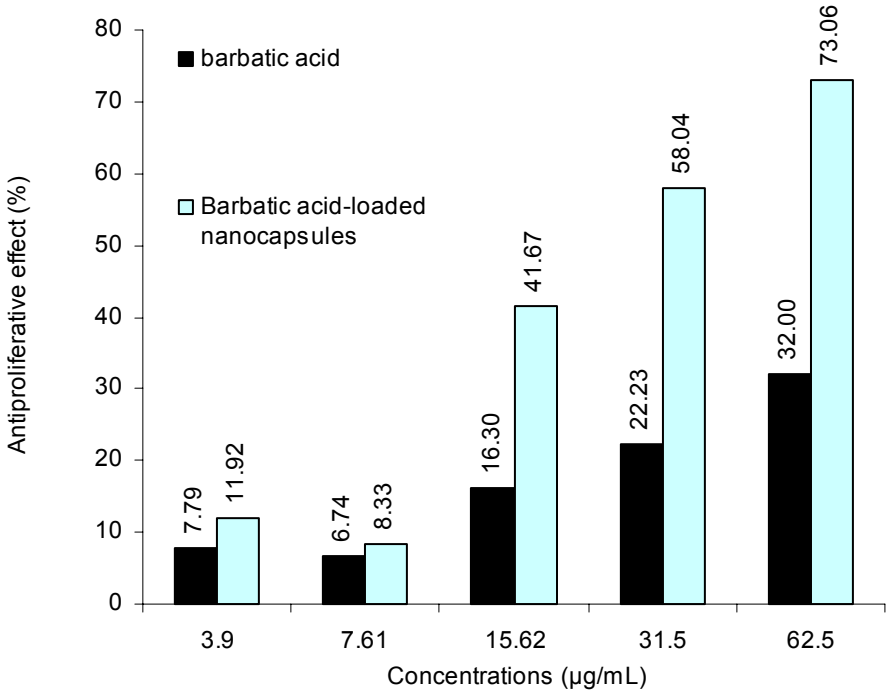

80
70
60
50
40
30
20
10
0
Antiproliferative effect (%)
barbatic acid
Barbatic acid-loaded nanocapsules
7.79
11.92
6.74
8.33
16.30
41.67
22.23
58.04
32.00
73.06
3.9
7.61
15.62
31.5
62.5
Concentrations (µg/mL)

**FIGURE 4.**

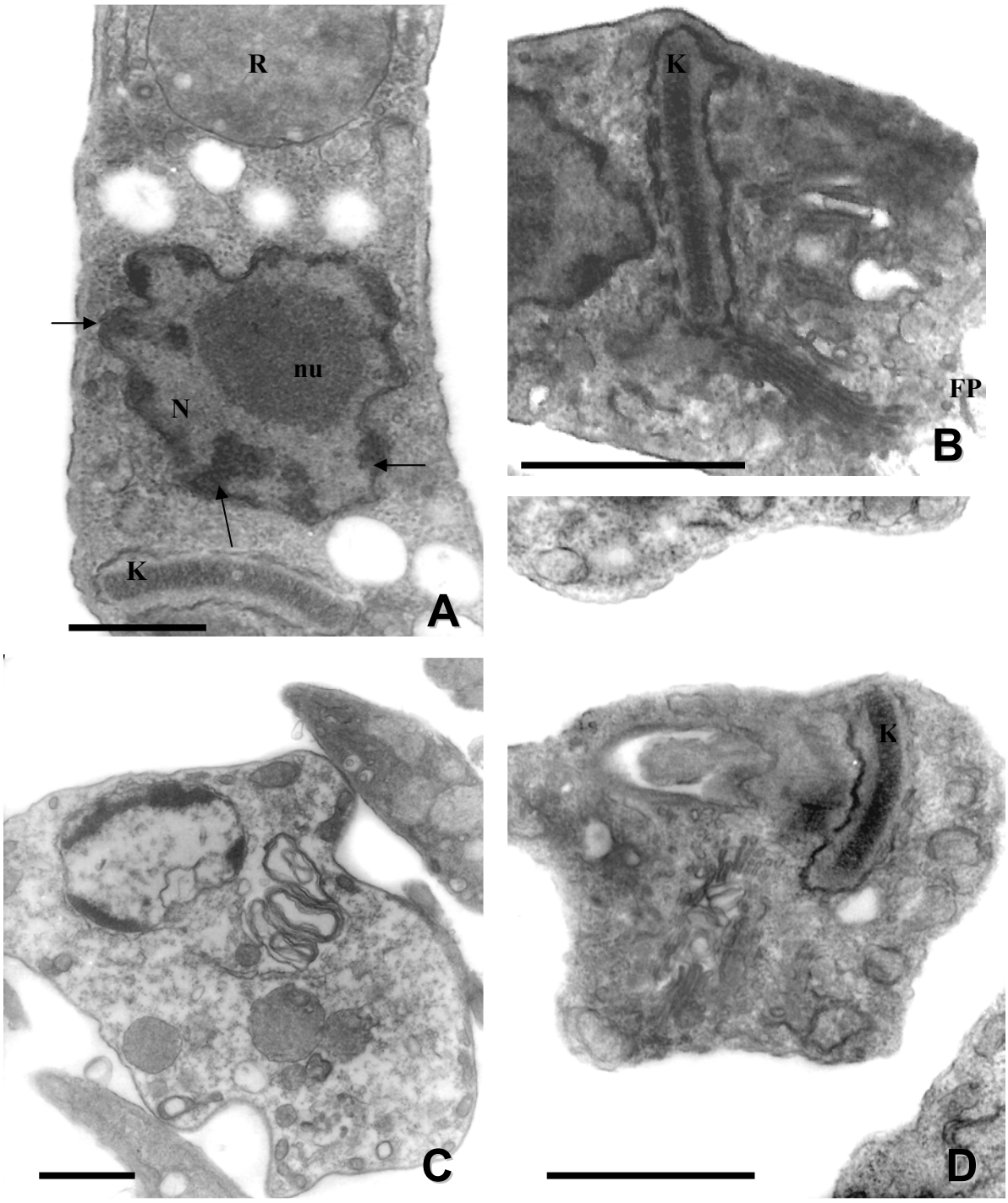
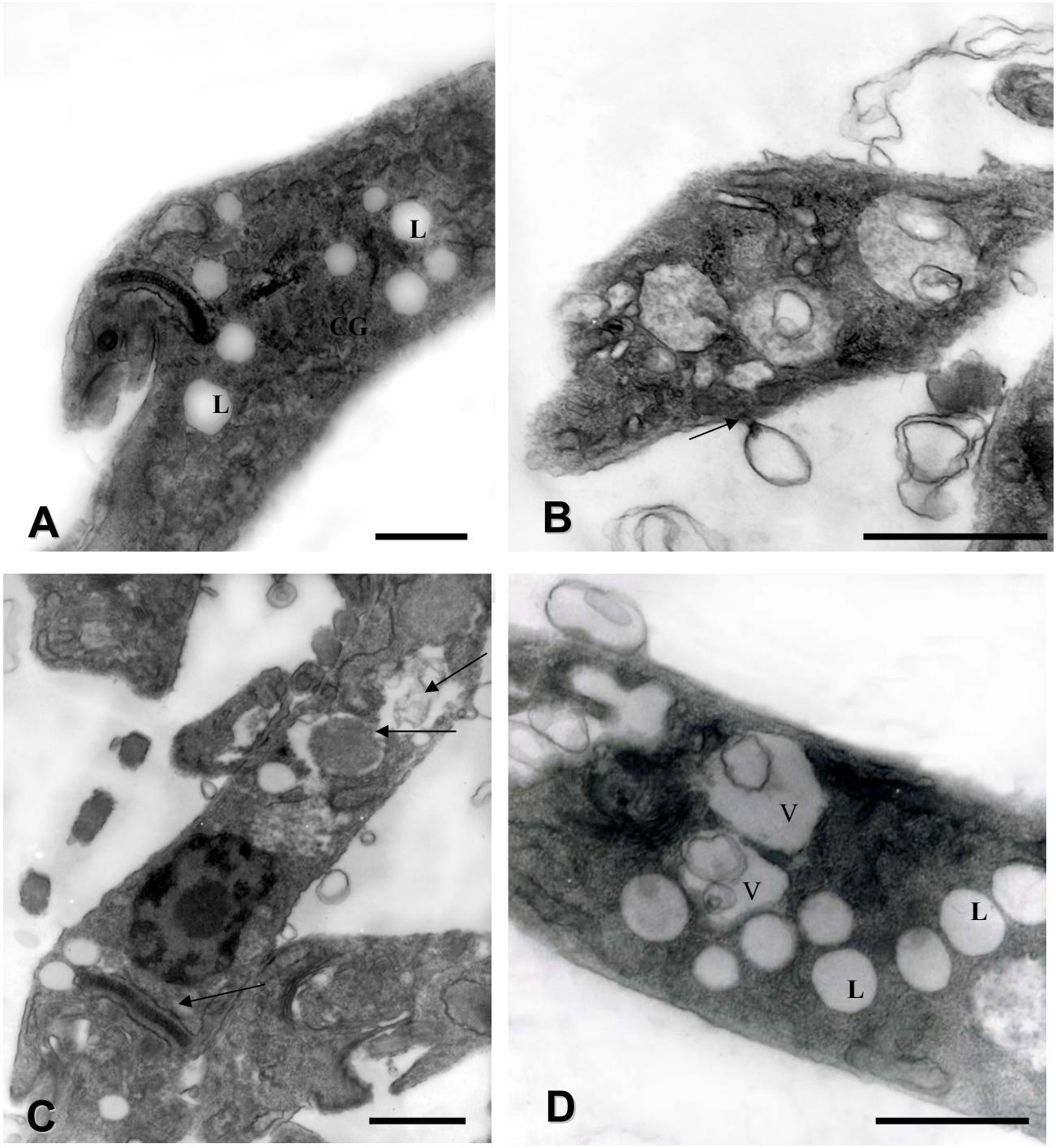


**FIGURE 5.**



# TABLES

**Table 1.**

| Barbatic acid Nanocapsule concentration (mg/mL) | Microscopic appearance | Macroscopic appearance | Centrifugation (2000 rpm- 1hour) | Mechanical stress (48h/140strockes/min 25ºC) |
|---|---|---|---|---|
| 0.5 | nanocapsules | Stable | Stable | Stable |
| 0.7 | nanocapsules | ppt | Stable | ppt |
| 1 | Presence of microcapsules | ppt* | ppt | ppt |

ppt = precipitation

**Table 2.**

| Days | Microscopic appearance | Macroscopic appearance | pH |
|---|---|---|---|
| 8 | nanocapsules | Stable | 7.2 |
| 15 | Presence of microcapsules | Stable | 6.9 |
| 30 | Presence of microcapsules | ppt | 7.2 |

ppt = precipitation

## FIGURES LEGEND

**Figure 1.** Chemical structure of barbatic acid (Huneck and Yoshimura, 1996).

**Figure 2.** Inhibitory effects of barbatic acid (A) and barbatic acid-loaded nanocapsules (B) epimastigote growth: control (◆); 3.9 µg/mL (■); 7.5 µg/mL (●); 15 µg/mL (×); 31.2 µg/mL (*) and 62.5 µg/mL (□). Data represent the mean value ± SD of three independent experiments.

**Figure 3.** The antiproliferative activity (%) of free (black column) and encapsulated barbatic (blue column) acid on Trypanosoma cruzi epimastigote forms after 24 hours of incubation

**Figure 4.** Effects of barbatic acid on the ultrastructure of *T. cruzi* epimastigotes: (A) epimastigote form grown in drug-free medium, showing a well preserved reservosome at the posterior end (R), the centrally located nucleus (N) with electrodense matrix, apparent nucleolus (nu) and patches of chromatin firmly adhered to the nuclear envelope membrane (arrows), and rod-shaped kinetoplast (K). Bar, 0.5 µm. (B ) detail of anterior region of control cell, showing a single Golgi complex located adjacent to flagelar pocket (FP) and kinetoplast (K). Bar, 1 µm. (C) aspect of epimastigote treated with barbatic acid at 83.45 µg/mL for 24 hours of incubation. To note the intense cellular disorganization, loose of the cytoplasm content (✱), as well as electrodense appearance of the nucleus (N) suggestive of cell apoptosis. Bar, 1µm. (D) barbatic acid-treated cell, showing the collapse of Golgi complex structure (CG); the kinetoplast (K) was apparently unaffected by the treatment. Bar, 1 µm.

**Figure 5.** Ultrastrucutral effects of barbatic acid-loaded nanocapsules on epimastigote forms: (A) detail of a cell parasite incubated with unloaded-PGLA nanocapsules, showing a great number of lipid-like inclusions (L) and displacement of Golgi complex (CG); epimastigote treated with 31.16 μg/mL for 24 hours of BARB-NC (B-D). Bar, 1μm. (B) detail of seriously damaged cell, showing the presence of large autophagocytic-like vacuoles containing membranous profiles. The membrane-bound structures, which seems to bud from plasma membrane (arrow) are commonly observed in these cells. Bar, 1 μm. (C) longitudinal section of epimastigote, showing the rupture of reservosomes (arrows) and appearance or electrolucent space in the cytoplasm. Again, the mictochondrial kinetoplast (arrows) was apparently unaffected by the treatment. Bar, 1μm. (D) large magnification of a parasite with BARB-NC showing the existence of a number of lipid-like inclusions (L) and large autophagic vacuoles (V). Bar, 0.5 μm.

**TABLES LEGEND**

**Table 1.** Accelerated stability testing of barbatic acid-loaded nanocápsulas.

**Table 2.** Long-term stability testing of barbatic acid-loaded nanocápsulas (0.5mg/ml).

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## 6. CONCLUSÕES

- A formulação mais estável de nanocápsulas de PLGA contendo ácido barbático foi observada com a concentração de 0,5 mg/ml.
- As suspensões permaneceram estáveis até quinze dias após sua preparação, observando a partir daí uma queda do pH e formação de microcápsulas.
- Observou-se queda na viabilidade celular das formas epimastigotas do *T. cruzi*, quando tratados com ácido barbático livre e nanoencapsulado, principalmente nas concentrações maiores.
- Uma maior atividade tripanossomicida foi observada nas primeiras 24h de incubação com ácido barbático nanoencaspulado.
- O efeito inibitório do ácido livre só pode ser observado após 48h.
- As nanocápsulas vazias não apresentaram efeito frente à proliferação celular, embora tenha se observado na ultra-estrutura mudanças na célula do parasita, como o acúmulo de lipídios.
- Tanto as nanocápsulas contendo ácido barbático como o ácido livre apresentaram modificações na ultra-estrutura, sendo que mais severas com o ácido encapsulado em PLGA.
- Na ultra-estrutura tanto o ácido livre quanto o encapsulado parece não afetar o cinetoplasto do parasita.
- A nanoencapsulação do ácido barbático mantém e potencializa sua ação parasitária nas primeiras 24 horas após a incubação.

# 7. ANEXO

## 7.1 Resumo submetido e aceito na SBBQ / 2007

## *IN VITRO* ACTIVITY AGAINST *TRYPANOSOMA CRUZI* OF BARBATIC ACID-LOADED NANOCAPSULES

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The aim of this study was to evaluate the effect of barbatic acid (BARB) on the viability and growth of *Trypanosoma cruzi* epimastigote forms, the ethiologic agent of Chagas' disease. In order to improve the BARB bioavailability, it was incorporated into poly(lactic-glycolic) nanocapsules (BARB-NC). Epimastigote forms of *T. cruzi* were incubated at different concentrations of free BARB or BARB-NC for 24, 48 and 72 hours. The $LD_{50}$ was evaluated by cell counting using Neubauer chamber. Parasites grown in drug-free medium was used as a control. Treatment of epimastigote with both BARB and BARB-NC resulted in a dose-dependent growth inhibition, with a value of $LD_{50}$/24h of 83.5 and 36.16 µg/ml, respectively. Incubation of parasites with 15.6 – 62.5 µg/ml leads to a significant increase of cell death soon after 24 hours of incubation for both free and encapsulated BARB. Furthermore, BARB-NC considerably increased trypanocidal activity as compared with unloaded BARB. Results showed that BARB-loaded nanocapsules can be used as a potential drug system against *T. cruzi*, being the poly-lactic-glycol a good carrier for this compound.

**Keywords**: Barbatic acid, *Trypanosoma cruzi,* PLGA nanocapsules

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Example 3: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

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