UNIVERSIDADE FEDERAL DO AMAZONAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

CONTRIBUIÇÕES PARA O CONHECIMENTO DA COMPOSIÇÃO QUÍMICA E ATIVIDADE BIOLÓGICA DE INFUSÕES, EXTRATOS E QUASSINÓIDES OBTIDOS DE *Picrolemma sprucei* Hook.f. (SIMAROUBACEAE).

RODRIGO CÉSAR DAS NEVES AMORIM

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Tese apresentada ao Programa Multi-Institucional de Pós-Graduação em Biotecnologia da Universidade Federal do Amazonas, como requisito para obtenção do título de Doutor em Biotecnologia, área de concentração Princípios Bioativos de Recursos Naturais Contra Patógenos de Doenças de Impacto Social no Brasil**.**

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Aprovado em 11 de setembro de 2009.

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À minha Esposa Márcia Rúbia, ao meu enteado Caio, à minha filha Marina e aos meus Pais e Irmãos por seu amor e carinho e apoio em todos os momentos.

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Resumo

Isobruceína B (**2**) e a neosergeolida (**5**) são quassinóides obtidos de *Picrolemma sprucei*. Essas substâncias possuem atividade antitumoral, antimalárica, anti-helmíntica, citotóxica, inseticida e leishmanicida in vitro já comprovadas. Neste trabalho, técnicas in vitro foram utilizadas para investigar uma variedade de atividades biológicas de **2** e **5** e dos derivados semi-sintéticos 1,12-diacetilisobruceína B (**21**) e 12-acetilneosergeolida (**22**). Essas substâncias foram avaliadas quanto à sua toxicidade geral frente a larvas do microcrustáceo *Artemia franciscana*, citotoxicidade frente a células tumorais humanas em colaboração com o laboratório de Oncologia da UFC, sob coordenação da Profa. Cláudia Pessoa, atividade larvicida frente a larvas do mosquito vetor da dengue *Aedes aegypti* e atividade antimalárica frente ao parasita causador da malária humana *Plasmodium falciparum*. **2** e **5** exibiram elevada atividade antimalárica frente à cepa multiresistente de *P. falciparum* K1 (CI_{50} 1,0-4,0 ng/mL). **2** e **5** (CL_{50} 3,2-4,4 mg/L) apresentaram letalidade bem mais elevada do que o derivado **22** frente a larvas de *A. aegypti*, ao passo que o derivado **21** se mostrou inativo. Ensaios biológicos também foram realizados com extratos e frações obtidos de frutos de *P. sprucei*. Um método baseado em CCD-densitometria foi desenvolvido para mensurar o teor de quassinóides nos frutos e frações destes frutos. Essas análises determinaram que apenas as frações obtidas em clorofórmio apresentam teores significativos de **2** e **5** e esses teores estão relacionados às atividades biológicas. Infusões dos caules de *P. sprucei* são utilizadas na Amazônia . Um método baseado em LC-(+)-ESI-MS/MS foi desenvolvido e aplicado para determinar o teor de **2** e **5** em infusões de *P. sprucei*. As concentrações de **2** e **5** nas infusões de caules são de 60,1 e 774 μg L^{-1}, respectivamente.

Abstract

Isobrucein B (**2**) and neosergeolide (**5**) are quassinoids which are obtained from *Picrolemma sprucei*. These compounds have proven *in vitro* antitumor, antimalarial, anthelminthic, cytotoxic, insecticide and leishmanicidal activities. Herein, *in vitro* techniques were used to investigate a range of biological activities of **2** and **5** and known semi-synthetic derivative 1,12-diacetylisobrucein B (**21**) and 12-acetylneosergeolide (**22**). These compounds were evaluated for general toxicity toward the brine shrimp species *Artemia franciscana*, cytotoxicity toward human tumour cells, larvicidal activity toward the dengue fever mosquito vector *Aedes aegypti* and antimalarial activity against the human malaria parasite *Plasmodium falciparum*. **2** and **5** exhibited the greatest antimalarial activity against multidrug-resistant *P. falciparum* K1 strain. **2** and **5** (LC_{50} = 3.2-4.4 mg/L) displayed greater lethality than derivative **22** (LC_{50} = 75.0 mg/L) toward *A. aegypti* larvae, while derivative **21** was inactive. Biological assays were also performed with extracts and fractions obtained from *P. sprucei* fruit. A TLC densitometry method was developed to mensurate the quassinoids content in fruits extracts and fractions. This analysis determinate that only chloroform fractions presents significative content of **2** and **5** and this data is related to the biological activities. Infusions of the stems of *P. sprucei* are used in the Amazon region as antimalarials. They contain **2** and **5**. An LC-(+)-ESI-MS/MS method was developed and applied to the determination of **2** and **5** in *P. sprucei* infusions. The concentrations of **2** and **5** in the stem infusion were found to be 60.1 and 774 μg L^{-1}, respectively.

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Lista de Abreviaturas e Siglas

CLAE - Cromatografia líquida de alta eficiência

CCD - Cromatografia em camada delgada

CCDP - Cromatografia em camada delgada preparativa

CI_{50} - Concentração inibitória 50%

CL_{50} - Concentração letal 50%

CPCS - Coordenação de Pesquisas em Ciências da Saúde

DL_{50} - Dose letal 50%

DMSO - Dimetilsulfóxido

i-PrOH - Isopropanol

IV - Infravermelho

Me - Metila

R_f - Fator de retenção

RMN - Ressonância magnética nuclear

RMN ^{1}H - Ressonância magnética nuclear de hidrogênio

RMN ^{13}C - Ressonância magnética nuclear de carbono

RP-18 - *Reverse phase* / fase reversa

UV Ultra violeta

TMS - Tetrametilsilano

Introdução

1. Levantamento Bibliográfico

1.1 - Família Simaroubaceae

A família Simaroubaceae contém aproximadamente 25 gêneros e 200 espécies. Dentre estas pelo menos 8 ocorrem na Amazônia Brasileira. Possui afinidades botânicas com as famílias Rutaceae, Burseraceae, Meliaceae e Cneoraceae. O principal uso comercial está relacionado à presença dos quassinóides (RIBEIRO et al., 1999, THOMAS, 1990). Quassinóides compõem um grupo de substâncias amargas que quimicamente são triterpenos degradados e altamente oxigenados em geral. De acordo com seu esqueleto básico, podem ser agrupados em 5 grupos contendo 18, 19, 20, 22 ou 25 carbonos (GUO et al., 2005).

1.2 - Gênero *Picrolemma*

O gênero *Picrolemma* foi descrito em 1862 por J. D. Hooker no "*Genera Plantarum*" com a espécie *P. sprucei* como tipo (Figura 1). Três outras espécies, *P. valdivia*, *P. pseudocoffea* e *P. huberi* (sin. *Cedronia granatensis* Cuatr. and *P. granatensis*) tem sido propostas. Destas, *P. huberi* é válida, *P. pseudocoffea* é uma sinonímia de *P. sprucei* e *P. valdivia* é incerta (CRONQUIST, 1944, THOMAS, 1990).

1.3 - *Picrolemma sprucei* Hook. f.

O nome vulgar caferana (café-rana ou café falso, na linguagem regional) se deve à semelhança desta planta com o cafeeiro (*Coffea arabica* L., Rubiaceae) (Figura 1). Outras espécies conhecidas como caferana são a *Tachia guianensis* Aubl. (Gentianaceae) e a *Quiina rhytidopus* Tul. (Quiinaceae) (PIO CORRÊA, 1926, SILVA; LISBÔA; LISBÔA, 1977).

Figura 1 – A - Planta Amazônica *Picrolemma sprucei* Hook. f, com florescência. B – Frutos do cafeeiro. C – Frutos de *P. sprucei*.

A importância da espécie *P. sprucei* e o potencial medicinal dos seus princípios ativos são grandes fora do Brasil, através de estudos efetuados por pesquisadores trabalhando em outros países da Amazônia, como Guiana Francesa, mas certamente o Brasil é o país com a maior população nativa dessa espécie (THOMAS, 1990).

No Brasil, acessos de *P.sprucei* foram detectados nos Estados do Pará, Rondônia e Amazonas, sendo que neste último distribuídos em todo o Estado (herbário do INPA, 2005). Em geral apresentam interações com insetos, principalmente formigas, que habitam o caule oco. Esse último é freqüentemente frágil e é a parte mais utilizada medicinalmente na Amazônia brasileira, sendo disponível para compra em feiras e através de raizeiros no interior do Estado (RIBEIRO et al., 1999).

Há grande interesse no estudo farmacológico *in vivo* dos quassinóides presentes na caferana e seus derivados semi-sintéticos, entretanto, a quantidade obtida nos processos de extração descritos previamente vem se mostrando como fatores limitantes para a continuação desses estudos. Aqui nós demonstramos um novo método para obtenção dos quassinóides da caferana em escala-grama, cuja purificação se baseia tão somente em cristalizações.

Estudos ao longo dos últimos 30 anos sobre os extratos de raízes, caules e folhas de *Picrolemma sprucei* revelaram a presença dos componentes antimaláricos neosergeolida e isobruceína B. Apesar do amplo uso de infusões de *P. sprucei* no tratamento da malária em toda a região Amazônica região, nenhum estudo demonstrou a atividade antimalárica destas infusões, bem como dos frutos da caferana

2. Objetivos

2.1 – Geral

- ✓ Contribuir para o conhecimento da atividade biológica e composição química de extratos de frutos, infusões de caules e quassinóides isolados da planta *Picrolemma sprucei* Hook.f. e preparar derivados semi-sintéticos a partir destes quassinóides.

2.2 – Específicos

- ✓ Aperfeiçoar a técnica de isolamento e purificação em escala-grama dos quassinóides neosergeolida e isobruceína B a partir de *P. sprucei*.
- ✓ Determinar o teor de quassinóides nos frutos e infusões de caules de *P. sprucei*.
- ✓ Estudar os quassinóides neosergeolida e isobruceina B, bem como extratos e frações do fruto em ensaios *in vitro* quantitativos para as seguintes atividades biológicas:
  - Antiparasitária (efeito sobre formas sanguíneas do parasita da malária humana e murina, *Plasmodium falciparum* e sobre o nematóide *Meloidogyne exigua*).
  - Tóxica (a larvas do crustáceo *Artemia franciscana*).
  - Citotóxica em linhagens de células tumorais humanas e avaliação do mecanismo de ação dos quassinóides usando células HL60 como modelo.
  - Inseticida (em larvas e imagos de *Aedes aegypti*).
  - Antimicrobiana (diversas linhagens de bactérias e fungos patogênicos).

- ✓ Estudar a reatividade desses quassinóides (em meios ácidos, alcalinos, redutores, oxidantes, etc.) e definir procedimentos para isolamento e purificação dos produtos.
- ✓ Estudar a atividade biológica de derivados dos quassinoides NS e IB nas atividades biológicas colocadas acima.

CAPÍTULO 1: *Picrolemma sprucei* Hook. f.: uso tradicional, princípios ativos e seus derivados semi-sintéticos, exploração comercial e econômica

Picrolemma sprucei Hook. f.: Uso Tradicional, Princípios Ativos e seus Derivados Semi-sintéticos, Exploração Comercial e Econômica

Picrolemma sprucei Hook. f.: Traditional Use, Active Principles and their Semi-synthetic Derivatives, Commercial and Economic Uses

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Resumo

Nesse revisão de literatura botânica, etnobotânica, etnofarmacológica, química e farmacológica, são enfatizadas as diversas atividades biológicas atribuídas pela medicina popular e por estudos científicos à planta Amazônica Picrolemma sprucei Hook. f. *(Simaroubaceae). Ênfase especial é dada aos conhecimentos atuais sobre os principais componentes químicos encontrados nessa espécie, da classe dos quassinóides: sergeolide (1), neosergeolide (5), isobrucaína B (2) e 15-desacetilsergeolide (4), que incluem atividade antimalárica, tóxica aguda, antitumoral, citotóxica, antifágica, larvicida/inseticida e fitotóxica. Também é incluída discussão das atividades biológicas de derivados (naturais e semi-sintéticos) dessas quassinóides com o intuito de revelar, além do potencial de P. sprucei como fonte de extratos para o aproveitamento fitoterapêutico, o potencial dessa planta como fonte de quassinóides bioativos para estudos de química medicinal e modificação estrutural.*

Unitermos

Picrolemma sprucei, Picrolemma pseudocoffea, quassinóide, isobrucaína B, neosergeolide, sergeolide, 15-desacetilsergeolide.

Abstract

In this review of the botanical, ethnobotanical, ethnopharmacological, chemical and pharmacological literature available concernig the Amazonian plant Picrolemma sprucei *Hook. f. (Simaroubaceae), the different biological activities attributed by traditional medicine and scientific studies are emphasized. Special emphasis is given to the status emphasis is given to the status of knowledge of the principal chemical components (quassinoids) found in this plant species: sergeolide (1), neosergeolide (5), isobrucein B (2) and 15-deacetylsergeolide which include antimalarial, acute toxic, antitumor, cytotoxic, antifeedant, larvicidal/insecticidal and phytotoxic properties. A discussion of the important derivatives (natural and semi-synthetic) and their biological activities intends to reveal the potential of this plant as a source of highly bioactive quassinoids for structural and activity medicinal chemistry studies, as well as the potential of P. sprucei as a source of extracts for phytotherapeutic use.*

Key words

Picrolemma sprucei, Picrolemma pseudocoffea, quassinoid, isobrucein B, neosergeolide, sergeolide, 15-deacetylsergeolide.

Introdução

A presente revisão tem como objetivo principal fazer uma síntese e análise crítica de informações bibliográficas das áreas de botânica, etnobotânica, química e farmacologia sobre a espécie *Picrolemma sprucei* Hook. f., os seus princípios ativos, bem como alguns derivados semi-sintéticos desses últimos. Onde pertinente, também estão incluídos informações e resultados inéditos das experiências e provenientes das pesquisas dos autores com essa planta. A importância dessa espécie como planta medicinal e o potencial medicinal dos seus princípios ativos são grandes fora do Brasil, através de estudos efetuados por pesquisadores trabalhando em outros países da Amazônia, como a França (Guiana Francesa), mas que certamente o Brasil é o país com a maior população nativa dessa espécie do planeta. Entre outros motivos que temos para a publicação do presente foi a exclusão ou não menção dessa espécie em uma das obras bibliográficas mais usadas e citadas no Brasil, o *Dicionário das Plantas Úteis do Brasil e das Exóticas Cultivadas*, de Pio Corrêa (1984), fato que deixou essa planta medicinal valiosa numa relativa obscuridade para pesquisadores e usuários brasileiros de plantas medicinais, não-familiarizados com plantas medicinais da região Amazônica. Outro fato importante é que na Amazônia brasileira se conhecem até 4 espécies, de famílias botânicas distintas, pelo nome "cafezana", entre elas, a *P. sprucei*. Pio Corrêa (1984) descreveu apenas uma dessas, a *Tachia guianensis* Aubl., da família Gentianaceae, uma planta, aliás, bastante rara ou "esporádica" na Amazônia brasileira e sem a ocorrência ubíqua de *P. sprucei*.

O nome cafezana (café-rana ou café falso, na linguagem regional) se deve à semelhança desta e algumas outras plantas na região com o cafeeiro (*Coffea arabica* L., Rubiaceae), em especial, isso é verdade no período de frutificação, quando a cafezana (*P. sprucei*) produz frutos verdes que se tornam alaranjados e posteriormente avermelhados ao amadurecerem em cacho localizado na ponta do caule. A planta é identificada pela sinonímia botânica *P. pseudocoffea* Ducke, nome que reconhece essa semelhança morfológica com o cafeeiro, em alguns textos (BERTANI et al., 2005; DUKE; VASQUEZ, 1994; LE COINTE, 1947; FANDEUR; MORETTI; POLONSKY, 1985; GRENAND; MORETTI et al., 1982; MILLIKEN, 1997; MORETTI; JACQUEMIN, 1987; MORS et al., 2000; POLONSKY; BHATNAGAR; MORETTI, 1984; SILVA; LISBOA; LISBOA, 1977; VIGNERON et al., 2005). O correto nome botânico de *P. sprucei* Hook. f., refletindo a precedência histórica na descrição dessa espécie, foi garantido por um perito nessa família botânica (THOMAS, W.; comunicação pessoal). A espécie *P. sprucei* pertence à família Simaroubaceae. Essa família se distingue de todas as demais pela presença nos seus tecidos de substâncias terpênicas altamente oxigenadas, conhecidas como quassinóides (THOMAS, 1990).

Morfologia Macroscópica

A *P. sprucei* é um arbusto que atinge até 2,5 m de altura, muito amargo em todas as suas partes. Apresenta folíolos cartáceos simétricos, verde-escuros e brilhantes. Quando em inflorescência apresenta flores de pétalas alaranjadas e estames amarelos. Os frutos são apocárpicos. A raiz central é espessa e penetrante, frequentemente alcançando 2 m ou mais de comprimento. A planta pode ser encontrada principalmente em regiões de platô, na capoeira e na mata de terra firme, no norte da América do Sul (LE COINTE, 1947; GRENAND; MORETTI; JACQUEMIN, 1987; RIBEIRO et al., 1999; SARAIVA et al., 2003; SILVA; LISBOA; LISBOA, 1977), já tendo sido registrada sua presença no Equador, Guiana Francesa, Peru, Colômbia e Venezuela (NYBG, 2005; MOBOT, 2005). No Brasil, acessos de *P. sprucei* foram detectados nos Estados do Pará, Rondônia e Amazonas, sendo neste último, distribuídos em todo o Estado, conforme dados obtidos do herbário do INPA – Instituto Nacional de Pesquisa da Amazônia. Em geral, a espécie apresenta interações com insetos, principalmente formigas, que habitam seu caule oco. Esta parte da planta é freqüentemente frágil, e é a parte mais utilizada medicinalmente na Amazônia brasileira, sendo disponível em feiras populares e por intermédio de raizeiros no interior do Estado (RIBEIRO et al., 1999).

Morfologia Microscópica

A anatomia foliar e caulinar de *P. sprucei* já foi estudada por Saraiva et al. (2003). As epidermes das folhas são glabras, apresentam células de paredes onduladas e estômatos anomocíticos. O mesofilo é dorsiventral com uma camada de células em paliçada e um parênquima lacunoso. Os feixes vasculares são

bicolaterais e apresentam idioblastos escuros de conteúdo tânico. No caule evidencia-se um parênquima cortical desenvolvido, com células de natureza esclerenquimática, isoladas ou reunidas em pequenos grupos, de paredes espessas. Internamente observa-se a região vascular com vasos xilemáticos isolados ou em grupos imersos em tecido fibroso, sendo o parênquima do tipo paratraqueal, vasicêntrico. Na região central encontra-se uma medula desenvolvida. A nervura mediana do terço médio do limbo foliar e o pecíolo possuem um conteúdo tânico e óleo-resinoso em algumas de suas células.

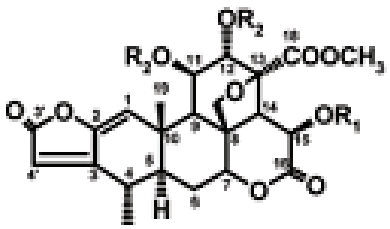
Estudos etnobotânicos e etnofarmacológicos

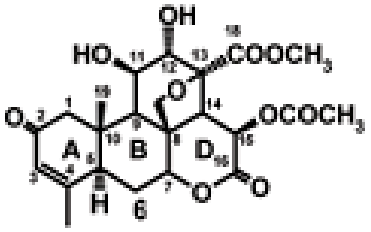
Bem conhecida por populações indígenas, *P. sprucei* tem sido utilizada há bastante tempo na medicina tradicional para o tratamento da malária (GRÉNAND; MORETTI; JACQUÉMIN, 1987). No Brasil, o chá das folhas e das raízes é usado no tratamento de gastrite, como febrífugo e antelmíntico (LE COINTE, 1947), sendo empregado no Peru para as mesmas finalidades (DUKE; VASQUEZ, 1994; MORS; RIZZINI; PEREIRA, 2000). No Estado do Amazonas, o uso popular de *P. sprucei* é bastante difundido, especialmente na região de Manaus (FERREIRA, 2000) onde é comum encontrar pedaços do caule de *P. sprucei* sendo comercializados no Mercado Municipal Adolpho Lisboa (Centro de Manaus), como antimalárico e febrífugo. Na Guiana Francesa, o chá do caule é utilizado pelos Créoles e *Palikur* uma vez ao dia, preferencialmente pela manhã, em combinação com *Fagara rhoifolia* Engl. para tratar a malária (GRÉNAND; MORETTI; JACQUÉMIN, 1987; MILLIKEN, 1997).

Um estudo etnofarmacológico realizado na Guiana Francesa com cento e dezesseis pessoas de cinco diferentes grupos e nacionalidades (Créoles, *Palikur*, Galibi, brasileira e européia) demonstrou que extratos alcoólicos preparados a partir de caules, folhas e raízes de *P. sprucei* são utilizados para o tratamento curativo da malária, sempre em combinação com outras espécies vegetais, preferencialmente *Quassia amara* L. (Simaroubaceae) e, em muitos casos, em combinação com drogas modernas. O extrato é obtido por maceração da planta em rum ou outra bebida alcoólica, por vários dias ou semanas. Um pequeno copo é tomado diversas vezes por semana. Foi constatado que, em muitos casos, as plantas medicinais são utilizadas por mais tempo do que as drogas modernas, podendo chegar até 15 dias de administração. Segundo a maioria dos entrevistados, as drogas modernas são capazes de curar a malária, entretanto, "as plantas seram". Dessa forma, o papel das drogas modernas é considerado essencial, porém complementar ao uso das plantas medicinais, as quais garantem a "cura verdadeira". Demonstrou-se ainda que os extratos de caferana, bem como de outras plantas, são utilizados para banhar o corpo, como uma forma de eliminar a febre, a malária e todas as impurezas. Já em relação ao tratamento preventivo, o extrato alcoólico de *P. sprucei* também foi utilizado sempre em combinação e preferencialmente com *Geissospermum* spp. (Apocynaceae) e *Q. amara*. Também foi constatado o uso do extrato etanólico de *P. sprucei* em banhos, como tratamento preventivo da malária (VIGNERON *et al.*, 2005).

Em um estudo subseqüente, Bertani e colaboradores (2005) demonstraram a capacidade do extrato aquoso de *P. sprucei* de inibir a formação da hemozoína, proteína derivada da digestão da hemoglobina, inibição esta que acarreta no acúmulo dos grupos heme, altamente tóxicos para o parasita. Ensaios *in vitro* demonstraram que o extrato de *P. sprucei* foi capaz de inibir o crescimento da cepa W2 (resistente à cloroquina) do parasita da malária humana *Plasmodium falciparum* (concentração inibitória mediana, CI_{50} 1,431 g/mL) e uma dose de extrato de 95 mg/kg de peso corporal inibiu em 77,5 % o crescimento de *P. falciparum in vivo* (cinco vezes menor do que a requerida pelo extrato de *Cinchona officinalis* L.). O extrato combinado de *P. sprucei*, *Q. amara* e *Geissospermum laevis* Miers (20 g de folhas frescas de *P. sprucei*, com 8 folhas frescas de *Q. amara* e 40 g de casca de *G. laevis* em 1,5 L de água fervente por 15 min) apresentou CI_{50} de 1,831 g/mL *in vitro* frente a *P. falciparum* e com inibição de 95,5% do crescimento da cepa 17X do parasita *Plasmodium yoelii yoelii* em ratos a uma concentração de 125 mg/kg, em apoio ao uso tradicional de *P. sprucei* no tratamento de infecções da malária. No ensaio de inibição da biomineralisação de ferriprotoporfirina IX (FBIT) os extratos de *P. sprucei* e de *Q. amara* foram os únicos mais ativos do que a *Cinchona calisaya* (controle positivo, CI_{50} 37 µg/mL), com CI_{50} de 30 e 51 g/mL, respectivamente. Por outro lado, o extrato aquoso preparado a partir das folhas de *P. sprucei* (como descrito acima) não apresentou nenhuma atividade no estágio intra-hepático do clone 1.1 da cepa 17X do parasita *P. yoelii yoelii*. Ao contrário do que havia sido levantado pela pesquisa etnofarmacológica, os testes não confirmaram a informação de que o extrato, na sua forma bruta, poderia ser utilizado em um tratamento preventivo contra malária.

Figura 1 – Quassinóides naturais encontrados na espécie *Picrolemma sprucei*.



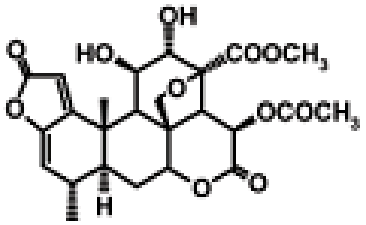


2

(1) $R_1 = H$; $R_2 = COCH_3$

(3) $R_1 = COCH_3$; $R_2 = COCH_3$

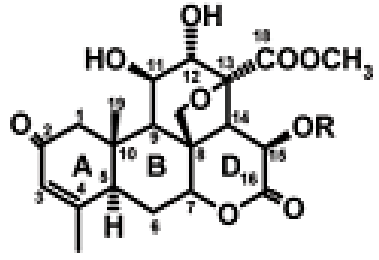
(4) $R_1 = H$; $R_2 = H$



5

Estudos químicos

Moretti et al. (1992) submeteram caules e raízes de *P. sprucei* encontrada na Guiana Francesa à eliminação de graxas e gorduras com hexano, seguida de extração aquosa exaustiva por infusão em água fervente. O extrato aquoso concentrado foi extraído continuamente com clorofórmio em sistema líquido-líquido e a partir da fração clorofórmica obtiveram-se os quassinóides sergeolida (**1**) e isobrucaína B (**2**) por cromatografia em fase normal (MORETTI et al., 1992). A acetilação da sergeolida (**1**) produziu a diacetilsergeolida (**3**). As folhas de *P. sprucei*, também da Guiana Francesa, foram submetidas ao processo de extração descrito acima foram isoladas a 15-desacetilsergeolida (**4**), além da sergeolida (**1**) e da isobrucaína B (**2**) (POLONSKY; BHATNAGAR; MORETTI, 1984). Dos caules e raízes de espécimes nativas da Amazônia brasileira, quando submetidos ao mesmo processo de extração, porém com pequenas modificações, foram isolados os quassinóides neosergeolida (**5**) e isobrucaína B (**2**) (SCHPECTOR et al., 1994; VIEIRA et al., 2000; SARAIVA, 2001).

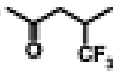


(2) R = $COCH_3$

(6) R = H

(7) R = 

(8) R = 

(9) R = 

(10) R = 

(11) R = 

Existem algumas outras espécies que apresentam a sergeolida (**1**) e a isobrucaína B (**2**) na sua composição, nenhuma daquelas sendo encontrada no Brasil. Por exemplo, a isobrucaína B (**2**) foi primeiramente isolada da espécie *Brucea antidysenterica* Mill. (KUPCHAN et al., 1975). Tischler e colaboradores (1992) também isolaram a sergeolida (**1**) e a isobrucaína B (**2**) da *Picrolemma huberi* Ducke (sin. *Cedronia granatensis* Cautrec.), e Bhatnagar et al. (1985) isolaram a isobrucaína B (**2**) de *Soulamea amara* Lam..

Atividade antimalárica dos quassinóides de *P. sprucei*

A sergeolida (**1**) apresenta elevada atividade antiplasmódio tanto *in vitro* quanto *in vivo*. Uma concentração de 50 ng/mL foi capaz de inibir em 94% o crescimento *in vitro* da cepa FUP de *P. falciparum* (sensível à cloroquina) em culturas com até 48 h com CI_{50} de 6 ng/mL (cerca de 3 vezes menor do que a da cloroquina). A isobrucaína B (**2**) apresentou atividade antimalárica *in vitro* cinco vezes menor do que a sergeolida (**1**). Nos testes *in vitro* realizados com as cepas resistentes à cloroquina 98/83, 95/83 e 97/83, sendo que esta última também se mostrou resistente à associação quinina/fansidar *in vivo*, a sergeolida (**1**) foi capaz de inibir significativamente o crescimento do parasita, a uma concentração de 6 ng/mL, enquanto que para a cloroquina um efeito inibitório idêntico somente foi possível com uma concentração cem vezes maior. Contagens de parasitas mostraram que a sergeolida (**1**), de forma semelhante à cloroquina, foi efetiva contra todos os estágios assexuados do parasita no sangue. Ensaios *in vivo* mostraram que a sergeolida (**1**) foi capaz de inibir a cepa NK65 de *Plasmodium berghei* em ratos, com DE_{50} (dose efetiva mediana) de 0,2 mg/kg/dia, ou seja, cinco vezes menor do que a da cloroquina (FANDEUR; MORETTI; POLONSKY, 1985). Esses resultados mostram a superioridade ou equivalência entre a atividade antimalárica *in vitro* e *in vivo* dos quassinóides e a cloroquina, agindo sobre cepas sensíveis.

Toxicidade e efeitos co-laterais de *P. sprucei* e seus componentes químicos

Como constatado por Vigneron e colaboradores (2005), muitas das drogas modernas, apesar de sua elevada eficiência, são tidas pela maioria da população como potencialmente tóxicas e apresentam forte incidência de danos nas funções hepáticas, aumentando os efeitos tóxicos da malária sobre o fígado. Para preparações contendo *P. sprucei*, reconhecida por todos os informantes como extremamente tóxica, a dose utilizada é de apenas uma pequena xícara de café (50 mL) três vezes por dia (20 g de folhas frescas em 1 L de água fervente por 10 min), por alguns dias e que esta não é recomendada para crianças. Além disso, na Amazônia brasileira, as preparações de *P. sprucei* não são recomendadas para grávidas, devido ao efeito fortemente abortivo. Por outro lado, chás fortes (ca. 10 g da casca triturado em infusão de 1 L) de água são usados para provocar o aborto. Devido à sua elevada toxicidade por injeção subcutânea a camundongos (dose letal mediana, DL_{50} 1,8 mg/kg), a sergeolida (**1**) não é indicada ao tratamento da malária de roedores (DE_{50} 1,7 mg/kg/dia). A isobrucaína B (**2**) apresentou DL_{50} de 5 mg/kg e, portanto, possui menor toxicidade a camundongos do que a da sergeolida (**1**) (FANDEUR; MORETTI; POLONSKY, 1985).

Atividade antitumoral da sergeolida, isobrucaína B e derivados

A sergeolida (**1**) apresentou elevada ação inibitória contra linhagens de células leucêmicas de camundongos (P388) (FANDEUR; MORETTI; POLONSKY, 1985). Por sua vez, a 15-diacetilsergeolida (**2**) apresentou apenas moderada atividade antileucêmica neste mesmo ensaio *in vitro*, apesar de demonstrar elevada atividade em testes *in vivo* (POLONSKY; BHATNAGAR; MORETTI, 1984). A sergeolida (**1**) e a isobrucaína B (**2**) foram utilizados em um "screening" de atividade antitumoral *in vitro* seguindo as orientações do NCI. Ambos produziram concentrações letais medianas (CL_{50}) na ordem de 10^{-5} a 10^{-6} M para a maioria das linhagens de melanoma e diversas linhagens de câncer de cólon, pulmão e outras linhagens de tumores sólidos (TISCHLER et al., 1992). Ôkano e colaboradores (1995) testaram a atividade antitumoral da isobrucaína B (**2**) e de mais 44 quassinóides. Dentre os dez mais potentes, destacaram-se aqueles contendo grupos carbonila e hidroxilas no anel A, uma ponte epoximetileno entre o C-8 e o C-13 e uma cadeia lateral contendo um grupamento éster em C-15 como são encontrados na isobrucaína B (**2**).

Fukamiya e colaboradores (2005), estudaram o

efeito de 63 quassinóides, dentre os quais a sergeolida (**1**), a isobrucaína B (**2**) e a 15-desacetilisobrucaína B (**9**), sobre a síntese de proteínas em células eucarióticas. Eles constataram que a isobrucaína B (**2**) e a 15-desacetilisobrucaína B (**9**) não apresentam qualquer efeito sobre a tradução de RNAm em células procarióticas (*E. coli*) até uma concentração final de 10 μM, ao passo que a isobrucaína B (**2**), a 15-desacetilisobrucaína (**6**) e a sergeolida (**1**) apresentam forte inibição da tradução de RNAm em células eucarióticas com CI_{50} de 0,16, 0,32 e 0,07 μM, respectivamente. Estes quassinóides apresentaram, ainda, elevada inibição da síntese protéica em células de carcinoma uterino HeLa com 97, 95 e 97% de inibição, respectivamente. Rahman e colaboradores (1997) realizaram um estudo de atividade antitumoral de derivados semi-sintéticos da isobrucaína B **5-10**. Todos os derivados ésteres demonstraram maior atividade antitumoral do que a apresentada pelo derivado desacetilado **5**. O composto **8**, contendo um éster alifático fluorado, foi o que apresentou a maior atividade, seguido pelos quassinóides contendo cadeias laterais aromáticas fluoradas **9** e **10**. A introdução de grupamentos fluorados na estrutura dos quassinóides aumenta tanto a lipofilicidade quanto a força das ligações, favorecendo, portanto, seus transportes até a célula, e protegendo-os da decomposição por oxidação enzimática. Muitos requerimentos estruturais necessários à atividade antineoplásica, exibida por diversos quassinóides, estão estabelecidas: A presença de um grupamento cetona α,β-insaturado no anel A; uma função éster no C-8 ou C-15 e uma ponte oximetileno entre o C-8 e o C-11 ou C-13 e um grupamento hidroxila no C-8 e/ou C-11 são características estruturais essenciais para uma forte atividade antineoplásica (BHATNAGAR; CARUSO; POLONSKY, 1987, KUBOTA et al., 1997, SPOHN; GRIECO; NARGUND, 1987). Ao contrário, um grupo hidroxila no C-13 e/ou C-15 e uma esterificação no grupo hidroxila no C-15 diminuem ligeiramente a atividade (KUBOTA et al., 1997). Os quassinóides encontrados em *P. sprucei* apresentam muitas dessas características estruturais associadas à atividade antitumoral.

Atividades antifágicas, fitotóxica e inseticida

Os quassinóides sergeolida (**1**), isobrucaína B (**2**) e 15-desacetilsergeolida (**3**) apresentaram potente atividade antifágica (inibição à vontade de se alimentar) e inibição do crescimento do verme *Helicthis virescens* f. (lagarta do broto do tabaco ou lagarta-da-maçã) e da praga do milho *Agrotis ipsilon* Hfn. (lagarta-rosca negra), sem provocar a morte dos insetos (LIDERT et al., 1987). Polonsky e colaboradores (1989) demonstraram que a isobrucaína B (**2**) apresenta forte atividade antifágica frente à espécie de pulgão *Myzus persicaes s.s.* (Sulzer) (Hemiptera, Aphididae), a uma concentração de 0,05%, com uma baixa fitotoxicidade a *Brassia campestris* var. Chinensis (L.) Makino (repolho branco chinês – pak choi), enquanto que a sergeolida (**1**) não se mostrou eficaz até uma concentração de 0,1%, porém causou danos consideráveis às folhas de repolho. Daldo e colaboradores (1995) demonstraram que, dentre 14 quassinóides naturais e 4 semi-sintéticos, a isobrucaína B (**2**) apresentou a maior atividade antifágica e inseticida frente a larvas no terceiro estágio da traça-das-crucíferas (*Plutella xylostella* L.). Os extratos metanólicos de cascas e raízes, bem como o extrato aquoso de raízes de *P. sprucei*, a uma concentração de 500 μg/mL, provocaram mortalidades de 57%, 74% e 44%, respectivamente, frente a larvas do mosquito *Aedes aegypti*. Entretanto, o extrato metanólico de folhas e os extratos aquosos de folhas e raízes de *P. sprucei*, na mesma concentração, não demonstraram capacidade larvicida contra *A. aegypti* (PÖHLIT et al., 2004).

Não existe cultivo de *P. sprucei*, até onde temos conhecimento. Tentativas de coletar frutos e efetuar germinação e produção de mudas em situações controladas tem tido sucesso limitado, levando à produção de algumas mudas, mas foi percebida a existência de dormência na germinação das sementes, que levam meses para germinar. Além disso, a produção esporádica, nem sempre anual, dos frutos e sementes é outro impedimento à produção sustentável. Tentativas de reproduzir a planta utilizando estaquia tiveram sucesso extremamente limitado, sendo que as estacas se mantiveram vivas nas condições de viveiro, mas não desenvolveram folhas e/ou raízes; ou seja, não geraram mudas de caferana, nas condições testadas até o presente momento (PÖHLIT, BARROS, LIMA JÚNIOR, resultados não publicados). O extrativismo é a atividade estabelecida para a *P. sprucei*, sendo um processo sustentável para atender a população local, a qual usa principalmente o caule. Experimentos preliminares com o transplante de mudas silvestres na região de

Manaus demonstraram que a planta se desenvolve rapidamente em áreas anoclaradas, apesar de ser freqüentemente encontrada em áreas de campinarana ou matas com pouca luz (PÖHLIT, BARROS, resultados não publicados) Essa observação é reforçada em áreas de queimadas e desmatamentos, em que a planta, inicialmente eliminada da superfície, desenvolve-se em áreas de pasto, em céu aberto, devido à resistência da sua raiz. No momento, em Manaus, entre outras instituições, estão sendo conduzidos experimentos na Universidade Federal do Amazonas (UFAM) e no Instituto Nacional de Pesquisas da Amazônia (INPA). A finalidade é o desenvolvimento de métodos biotecnológicos baseados em cultura de células de *P. sprucei*, visando reproduzir a planta através de plântulas ou outros meios. Também estão em andamento estudos sobre a velocidade de recrescimento de caules/planta X área, com o fim de avaliar a sustentabilidade e os parâmetros de produção associados ao extrativismo, o único método de fornecimento existente para essa valiosa espécie Amazônica.

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CAPÍTULO 2: Inibição *in vitro* do *Plasmodium falciparum* por agentes quimioterápicos derivados de plantas medicinais Amazônicas

In vitro inhibition of *Plasmodium falciparum* by substances isolated from Amazonian antimalarial plants

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In the present study, a quassinoid, neosergeolide, isolated from the roots and stems of Picrolemma sprucei *(Simaroubaceae), the indole alkaloids ellipticine and aspidocarpine, isolated from the bark of* Aspidosperma vargasii *and* A. desmanthum *(Apocynaceae), respectively, and 4-nerolidylcatechol, isolated from the roots of* Pothomorphe peltata *(Piperaceae), all presented significant in vitro inhibition (more active than quinine and chloroquine) of the multi-drug resistant K1 strain of* Plasmodium falciparum. *Neosergeolide presented activity in the nanomolar range. This is the first report on the antimalarial activity of these known, natural compounds. This is also the first report on the isolation of aspidocarpine from* A. desmanthum. *These compounds are good candidates for pre-clinical tests as novel lead structures with the aim of finding new antimalarial prototypes and lend support to the traditional use of the plants from which these compounds are derived.*

Key words: neosergeolide - ellipticine - aspidocarpine - 4-nerolidylcatechol - *Pothomorphe peltata* - *Picrolemma sprucei* - *Aspidosperma* spp.

Malaria is the main cause of economic loss and high morbidity in the world today and continues to be endemic to tropical regions such as the Amazon. In the Brazilian Amazon, 1.6 million positive plates (thick smears) in a total of 8 million diagnostic tests for malaria were registered from January 2004 to February 2007 (Ministério da Saúde, Sivep-Malaria 2007). The lack of an effective vaccine and the increasing expansion of strains of *Plasmodium falciparum* presenting resistance towards commonly used, low-cost antimalarials make control of this disease difficult (Olliaro & Bloland 2001, Wellems & Plowe 2001, Vieira et al. 2001, 2004, Gonzales et al. 2003, Alecrim et al. 2006). As a result, the World Health Organization (WHO 1978, 1995) has been promoting research on natural product based drugs for treatment of disease and many plant species have been evaluated for antimalarial activity (Weniger et al. 2004). In these studies, emphasis has been on the discovery of lead compounds for drug development (Gundidza & Chinyanganya 1999). The rational search for active substances in medicinal plants is a very promising and cost-effective strategy for antimalarial drug discovery. This approach benefits from the accumulated knowledge of the curing capacity of plants possessed by inhabitants of malaria endemic regions and permits the extensive evaluation of natural products derived from these sources (Campbell et al. 1997, 1998, 2000, Carvalho & Krettli 1991, Carvalho et al. 1991, Brandão et al. 1992, 1997, Krettli et al. 2001, Andrade-Neto et al. 2004a,b).

This triage of useful and effective plants is at the heart of traditional medicinal knowledge and is an extremely important source of therapeutic compounds in use today. Important semi-synthetic, low-cost, highly effective antimalarial drugs such as the quinolines (chloroquine, mefloquine, primaquine, etc.) and artemisinin derivatives (sodium artesunate, artether, artemether, etc.) owe their initial discovery to the isolation and structural identification of antimalarial natural products (quinine and artemisinin, respectively) from traditionally used antimalarial plant species (*Cinchona* spp. by Amerindians in Peru and *Artemisia annua* in China, respectively) (Rosenthal 2003). Recent studies on traditionally used antimalarial remedies have revealed plants which produce indole and isoquinoline alkaloids, sesqui-, di- and triterpenes, flavonoids and other substances presenting proven in vitro activity against *P. falciparum* (Frederich et al. 1999, Phillipson 1999, Muhammad et al. 2004).

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Brazilian flora, especially the vast Amazon forest, is a large, mainly unexplored source of molecules with pharmacological potential. Some examples of substances derived from plants used by Brazilian indians are pilocarpine, extracted from the leaves of jaborandi (*Pilocarpus jaborandi*), emetine, obtained from the roots of ipeca (*Cephaelis ipecacuanha*), guarana seeds (*Paullinia cupana*), rich in caffeine, used as stimulant, and curares, extracted from the leaves of *Chondodendron* spp., used in anesthetic techniques.

Despite the richness of our flora, the role of Brazil in the global production of drugs has been that of a mere supplier of starting materials for international industry. This industry has grown steadily in recent years and is now estimated at US$ 22 billion each year in phytotherapeutic agents and plant-derived pharmaceuticals (Pinto et al. 2002). However, research in Brazil on chemical, biochemical, and genetic resources must be intensified. Also, academy-industry partnerships must increase in number and must also be accompanied by flexible financing from private and public sectors. Through these and other measures, the identification of bioactive compounds can in principle be optimized, leading to greater profits from the commercialization of plant materials and also lead to a greater degree of sovereignty over our biodiversity.

Research on new antimalarials from natural products involves coordinated scientific effort on the part of different professionals. These professionals generally represent distinct academic disciplines, most importantly, botany, natural product, and synthetic chemistry, pharmacology, parasitology, and molecular biology. Groups with these characteristics can in the short and long run produce sound knowledge of the chemical, pharmacological, and biological diversity. Based on scientifically sound facts, the most promising agents for further clinical and industrial development can be identified.

In this work, the pharmacological potential of several substances isolated from traditionally used antimalarial plants was evaluated through screening for in vitro inhibition of human malaria parasite species *P. falciparum*. The ultimate goal of this work is to identify new classes of antimalarial substance which may serve as prototypes for the development of drug leads having novel mechanisms of action.

MATERIALS AND METHODS

Plant material, extraction, and chemical constituent isolation - The plants from which the substances under study were isolated are traditionally used for the treatment of malaria and fever in the Amazon region and are known to locals as *caferana* (*Picrolemma sprucei* Hook.f., Simaroubaceae), *amargoso*, *araracanga* (*Aspidosperma desmanthum* Benth. ex Müll. Arg., Apocynaceae), *amarelão* (*Aspidosperma vargasii* A. DC.), and *cuapeba-do-norte* (*Pothomorphe peltata* L., Piperaceae). All plant materials were collected in the state of Amazonas in the locations specified below. Voucher specimens are deposited at the Instituto Nacional de Pesquisas da Amazonia and Universidade Federal do Amazonas Herbariums. Plants were dried in the shade or laboratory and milled prior to extraction. Structural elucidation of isolated compounds was performed by analysis of 1-D / 2-D NMR, mass, infrared and ultraviolet spectral data and comparison to spectral data available in the literature.

Isolation of 4-neolitdylcatechol (1) from P. peltata - Cuapeba-do-norte was cultivated at Embrapa Amazonia Ocidental (Manaus) and harvested. Roots (150 g) were extracted with a 1:1 mixture of $CHCl_3$/EtOH (3 × 150 ml; 15 min each) in an ultrasound bath. After total evaporation, the extract (19.5 g; 13%) was chromatographed on silica gel using a 9:1 mixture of $CHCl_3$/EtOH 9:1 which yielded pure 1 (8.6 g, 44.1% w /w based on extract, 5.7% based on dry weight of plant.

Isolation of neosergeolide (2) from P. sprucei - Caferana was collected in the town of Silves. Roots and stems (6.5 kg) were degreased with hexanes in a soxhlet apparatus then repeatedly extracted with water using the same equipment. Continuous liquid-liquid extraction of the resulting concentrated H_2O extract with $CHCl_3$ was then performed. The procedure described up to this point was essentially that described by Moretti et al. (1982) for the isolation of other quassinoids from this plant. We developed a method which obviates the need for a chromatography step. The concentrated chloroform extracts (35.1 g) were dissolved in a minimum of hot water and acetone (2:1). The resultant precipitate was fractionally recrystallized to give pure neosergeolide (2) (685.4 mg, 0.011% based on dry weight of plant).

Isolation of ellipticine (3) from A. vargasii - Amarelão was collected at Inpa's Adolpho Ducke Reserve in Manaus. Bark (1.30 kg) was macerated in EtOH/1% NH_4OH (aq.). After filtration, evaporation of solvents and freeze-drying, extract (56 g) was obtained which was dissolved in ethyl acetate and partitioned with dilute, aqueous hydrochloric acid. The aqueous layer was basified to pH 8 and then extracted with chloroform. After separation of layers and total evaporation, an alkaloid-rich fraction was obtained (8.02 g). A portion (7 g) of this fraction was sequentially chromatographed on silica (CC, then TLC) using different eluentes to yield ellipticine (3, 68.2 mg, 0.0052% based on dry weight of plant).

Isolation of aspidocarpine (4) from A. desmanthum - Collection and extraction of *amargoso* (*araracanga*), as well as isolation and purification of 4, was essentially the same as described above for 3, wherein bark (1.2 kg) yielded ethanol extract (35 g) which was partitioned and yielded an alkaloid rich pH 8 fraction (1.45 g). Sequential normal-phase chromatography on a portion (1.40 g) of this fraction yielded aspidocarpine (68.5 mg, 0.0057% based on dry weight of plant).

Parasite culture and in vitro antimalarial tests - Chloroquine, pyrimethamine, and cycloguanil resistant *P. falciparum* strain K1 was acquired from MR4 (Malaria Research and Reference Reagent Resource Center, Manassas, Virginia, US) and was used in the in vitro tests. Parasites were maintained in continuous culture in A+ human erythrocytes, using RPMI medium supplemented with 10% human serum, as described by Trager and Jensen

(1976). The antiparasitic effect of the compounds was measured by growth inhibition percentage as described by Carvalho and Krettli (1991). Briefly, trophozoite-stages in sorbitol-synchronized blood (Lambros & Vanderberg 1979) were cultured at 1-2% parasitaemia and 2.5% hematocrit and then incubated with the plant extracts or isolated compounds (maximum 1 mg/ml in serial dilutions), diluted with 0.02% final concentration of DMSO in culture medium (RPMI 1640) for a total of 48 h at 37°C. A positive control with reference antimalarial drug (chloroquine and quinine) in standard concentrations (Rieckmann et al. 1978; WHO 2001) was used in each experiment. The stock solutions were further diluted in complete medium (RPMI 1640 plus 10% human serum) to each of the used concentrations (0.0001 up to 100 µg/ml in seven dilutions). The half-maximal inhibitory (IC_{50}) responses as compared to the drug-free controls were estimated by interpolation using Microcal Origin® software. Each duplicate experiment was repeated three times and blood smears were read blind.

Statistical analysis - The data of in vitro antimalarial tests were analyzed with the Biostat 1.0 MCT-CNPq software package using Anova and Student's *t*-test.

RESULTS

The results of the in vitro tests with compounds obtained from plant extracts against multidrug-resistant *P. falciparum* K1 strain are presented in the Table. The IC_{50} of compounds ranged from 2.0 nM to 0.67 µM. Neosergeolide (2), a known quassinoid which has previously been isolated from *Picrolemma sprucei* but for which no data on antimalarial activity has been previously reported, showed significantly higher activity (IC_{50} = 2.0 nM) than did the other compounds tested. Fig. 1 illustrates the dose-response curve for this quassinoid showing a tendency of standard curve; this analysis was performed for all tested compounds. Ellipticine and aspidocarpine are known indole alkaloids for which antimalarial activity has not apparently been previously described. Both presented significant inhibition of parasite growth (IC_{50} = 73 and 19 nM, respectively). 4-Nerolidylcatechol, a metabolite found in *Pothomorphe peltata* for which no data is available as to antimalarial activity, presented good parasite inhibition (IC_{50} = 0.67 µM). The in vitro sensitivity of the *P. falciparum* strain to the compounds tested was similar and reproducible in assays in duplicate on separate occasions.

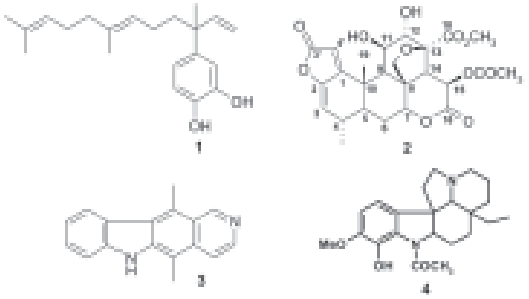

Fig. 1: structures of isolated antimalarial compounds.

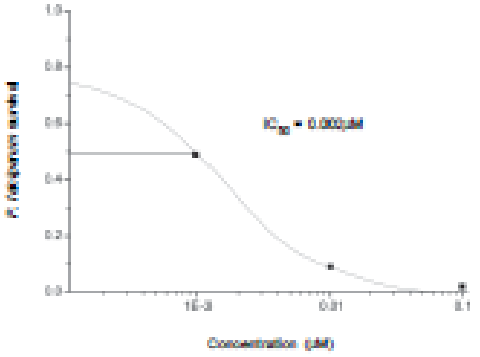

Fig. 2: illustrative dose-response curve for multi-drug resistant *Plasmodium falciparum* K1 strain in the presence of different concentrations of the quassinoid neosergeolide (2) tested with IC_{50} value in representative experiment; confidence interval (95% CI) = 0.00–0.01. Statistical analysis among additional assays showed: standard error = 0.0007; $p = 0.10$.

TABLE

The half-maximal inhibitory concentrations (IC_{50}) of isolated substances from *Pothomorphe peltata*, *Picrolemma sprucei*, *Aspidosperma vargasii*, and *A. desmanthum* towards *Plasmodium falciparum* (K1 strain)

Compound name	Structural class	Plant species/source	Mean IC_{50} values[a]	
			µg/ml	µM
Neosergeolide	Quassinoid/terpenoid	*Picrolemma sprucei*	0.001	0.002
Ellipticine	Indole alkaloid	*Aspidosperma vargasii*	0.018	0.073
Aspidocarpine	Indole alkaloid	*Aspidosperma desmanthum*	0.007	0.019
4-Nerolidylcatechol	Phenylpropanoid/terpenoid	*Pothomorphe peltata*	0.21	0.67
Chloroquine diphosphate salt	Quinoline	Synthetic commercial standard	0.46	0.89
Quinine salt	Quinoline alkaloid	Natural commercial standard	0.004	0.012

a: mean values in representative assay. All experiments were performed in duplicate.

DISCUSSION

Plants of the family Simaroubaceae are widely used in traditional medicine for the treatment of malaria, cancer, dysentery, and other diseases in countries around the world (Okano et al. 1990, Muhammad et al. 2004). Quassinoids are a group of degraded triterpenes found in the family Simaroubaceae, that show many biological activities such as antitumor (Fukamiya et al. 2005), antifeedant (Daido et al. 1995), phytotoxic (De Feo et al. 2003), antiviral (Apers et al. 2002), and antihelmintic (Nunomura et al. 2006). The antimalarial activity of some quassinoids like brusatol, glaucarubinone and quassin has been demonstrated previously (Wright 2005).

An ethnopharmacological study in French Guyana showed that *P. spruceí* root, stem and bark alcohol extract is used in local traditional medicine as a curative treatment for malaria in association with *Geissospermum* spp. (Apocynaceae) and *Quassia amara* (Simaroubaceae) or modern drugs (Vigneron et al. 2005). In a subsequent study, Bertani et al. (2005) demonstrated that *P. spruceí* water extract can inhibit hemozoin formation. In vitro assays demonstrated the antimalarial activity of this extract against the chloroquine resistant *P. falciparum* strain W2. Sergeolide and isobrucein B are quassinoids which have been isolated previously from *P. spruceí* and exhibit high antiplasmodial activity. Against chloroquine-sensitive FUP strain, sergeolide exhibited an IC_{50} which was five times less than that of isobrucein B and three times less than the that of chloroquine in the same strain. In vivo assays demonstrated that sergeolide was capable of inhibiting *P. berghei* strain NK65 with an ED_{50} of 0.2 mg/kg/day, five times less than chloroquine (Fandeur et al. 1985).

Our data show that the quassinoid isolated from the roots and stems of the *P. spruceí* was more active than quinine and chloroquine; with activities in the micromolar ranges comparable to recently reported results. Several quassinoids are known to inhibit the growth of *P. falciparum* in culture at nanomolar concentrations (Kuo et al. 2004). The quassinoids orinocinolide and simalikalactone D, isolated from the root bark of *Simaba orinocensis* were found to be potent in vitro against *P. falciparum* clones D6 and W2 (Muhammad et al. 2004). Research has also revealed quassinoids which are 4 and 12 times more active in vivo (via oral) against rodent malaria parasite (Phillipson et al. 1993) than chloroquine and artemisinin, respectively (Kim et al. 2000). Despite these antimalarial activities, quassinoids usually present toxicity due principally to protein synthesis inhibition and it is likely that parasite and host cell ribosomes are too similar to allow for the development of selective inhibitors (Wright 2005). Some structural requirements, like an α,β-unsaturated ketone in the A ring, an epoxymethylene bridge in the C ring and an ester function in C-15 are considered very important for the antimalarial activity presented by quassinoids (Phillipson et al. 1986, Amorim & Pohlit 2006).

Two morphologically similar species of *Pothomorphe*, namely *P. peltata* and *P. umbellata*, occur widely in Brazil. Both are known by the popular name *caapeba*, among other names, and are used in traditional medicine for the treatment of malaria. Both plants produce the secondary metabolite of mixed terpene and phenylpropanoid biosynthetic origin, 4-nerolidylca-techol, which was evaluated in the present study. Qualitative tests have shown the presence of this compound in the root, leaf and inflorescences of *P. peltata* and literature would seem to suggest a similar distribution in *P. umbellata* (Pinto 2002).

Several studies on the in vitro and in vivo antimalarial activities of *P. umbellata* and *P. peltata* have been reported. Amorim et al. (1986) evaluated the ethanol extracts of *P. umbellata* (leaves) and *P. peltata* (whole plant) against *P. berghei* in vivo. Reductions in parasitemia of 66, 55, and 28% were observed for the *P. umbellata* extract, whereas no activity was observed for *P. peltata* extract. In a similar study, Amorim et al. (1988) evaluated leaf ethanol extracts of these same species by subcutaneous and oral administration and found that *P. umbellata* leaf extract significantly reduced blood parasite levels at different doses whereas *P. peltata* leaf extract was inactive at 500 mg/kg in both tests. On the other hand, Adami (1995) evaluated *P. peltata* and *P. umbellata* leaf hexane and methanol extracts in vivo through oral and subcutaneous administration in *P. berghei* infected mice and found that these extracts were inactive against blood forms of *P. berghei*. These results lead Ferreira-da-Cruz et al. (2000) to conclude that the oral or subcutaneous administration of plant extracts in *Plasmodium berghei* infected rats was not effective at detecting the antimalarial activity of these plants.

Sala-Neto et al. (1992) tested the in vivo and in vitro antimalarial activity of *P. peltata* leaf, root and stem water extract using a new method. Briefly, this method involved oral administration in adult rats via gavage tube (6 x 6 ml) for 2 days. After this period of treatment, the rats were bled and blood sera were tested in vitro in microcultures of *P. falciparum* using tritium-labeled hypoxanthine incorporation for parasite quantification. In vitro *P. falciparum* inhibition (49%) was observed for serum obtained from rats inoculated with *P. peltata* water extract versus controls. From the results of this and other experiments, differences in *P. berghei* and *P. falciparum* blood-stage biology might be thought to be responsible for the lack of in vivo activity observed for *Pothomorphe* spp. extracts by some authors.

Adami (1995) also evaluated *P. peltata* and *P. umbellata* leaf hexane and methanol extracts in vitro in human malaria parasite species *P. falciparum*. The methanol extracts of both species presented greater inhibition of *P. falciparum* growth than the hexane extracts. In a more recent study, Atindehou et al. (2004) observed the in vitro antiplasmodial activity of *P. umbellata* leaf ethanol extract (IC_{50} 3.7 µg/ml) in chloroquine and pyrimethamine resistant *P. falciparum*. We obtained a similar result for the alcohol root extract of *P. peltata* in vitro in the K1 strain of P. falciparum. In preliminary work, 4-nerolidylcatechol (1) was shown to be active against *P. falciparum* in vitro (Pinto 2002).

The screening of natural products provides the chance to discover new molecules of unique structure with high

activity and selectivity which can be further optimized by semi- or fully synthetic procedures (Holzgrabe & Bechthold 1999).

Alkaloids are one of the most fascinating classes of natural products, providing many drugs for human use (Phillipson et al. 1993, Kayser et al. 2003). In general, indole alkaloids are a class of compound having a range of biological activities, including antibacterial, trypanocidal, leishmanicidal and anticancer (Sakamoto-Hojo et al. 1988, Delorenzi et al. 2001, Kuo et al. 2004, Ferreira et al. 2006, Tanaka et al. 2006). The antiplasmodial activity of monoterpene indole alkaloids has been investigated (Wright et al. 1994, Iwu et al. 1994). Promising results have been obtained previously by others for aspidospermidine structural analogues isolated from *A. pyrifolium* and *A. megalocarpon* to Nigerian chloroquine-sensitive and a Camaroon chloroquine-resistant (FcM2) strain of *P. falciparum*. In the chloroquine-resistant strain, apidospermine, 10-methoxyaspidospermidina and N-formylaspidospermidine presented, after 24 h, IC_{50} of 16.3, 19.5 and, 16.1 µM, respectively, whereas after 72 h, IC_{50} were 3.8, 3.2, and 5.6 µM, respectively. In the chloroquine-sensitive strain, after 24 h, IC_{50} were 11.0, 13.1, and 22.0 µM, respectively, and after 72 h, 4.6, 5.1, and 5.9 µM, respectively (Mitaine-Offer et al. 2002). Here, the isolated monoterpene indole alkaloids ellipticine (3) and aspidocarpine (4) were more active. The activities against K1 strain were of the same order as those observed for the terpenoid-phenylpropanoid compound 4-nerolidylcatechol (1).

Those compounds or chemical groups have already shown potential as new drug leads or may have an impact on future drugs. Further studies should explore these compounds as a prototype for an antimalarial aimed at the *P. falciparum* mult-resistant parasites.

Adaptation of the protocol cited above to high-throughput platforms, as well as implementation of modern indirect methods for the quantification of in vitro parasite growth, such as fluorimetry (Smilkstein et al. 2004) are underway and will be essential for an increase in the scale and dynamism of studies on antimalarial plants, isolated natural substances and their semi-synthetic derivatives, potentializing a process of continuous screening in the near future.

Additionally, stabilization of geographically specific *P. falciparum* populations in continuous in vitro culture is underway and should permit investigations into the real susceptibility profile of these regional parasites to the active substances and plant extracts which present promising inhibitory concentrations. It is our hope that knowledge of this regional profile can be useful for the identification, based on sound experimental evidence, of the most important and effective medicinal plants for development of new and effective antimalarials for local use. Furthermore, simultaneous studies on the macromolecular profiles of these parasites in association with analysis of genetic resistance markers (Mu et al. 2007) should contribute to the elucidation of possible mechanisms of resistance of the parasites to the natural products tested as well as aid in the discovery of new targets (and/or new mechanisms of action) for antimalarial chemotherapy.

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CAPÍTULO 3: Isolamento da isobruceína B e da neosergeolida de *Picrolemma sprucei* Hook. f. em escala-grama.

ACTA AMAZONICA

Gram-scale isolation of isobrucein B and neosergeolide from *Picrolemma sprucei* Hook. f.

Ellen Cristina Costa da SILVA[1], Rodrigo César das Neves AMORIM[2], Wanderli Pedro TADEI[3], Adrian Martin POHLIT[4]

ABSTRACT

Quassinoids neosergeolide and isobrucein B, obtained from *Picrolemma sprucei*, have proven *in vitro* antitumor, antimalarial, anthelminthic, cytotoxic, insecticide and leishmanicidal activities. There is interest in the *in vitro* pharmacological study of these natural compounds and their semi-synthetic derivatives, however, the quantities obtained in previous extraction processes have been shown to be a limiting factor for continuation of these studies. Herein, we describe a method for obtaining grams of these quassinoids whose purification relies only on recrystallization.

KEYWORDS: Caferana, Simaroubaceae, Quassinoid, Recrystallization.

Isolamento de isobruceína B e neosergeolida de *Picrolemma sprucei* Hook. f. em escala-grama

RESUMO

Os quassinóides neosergeolida e isobruceína B, obtidos de *Picrolemma sprucei*, possuem atividades antitumoral, antimalárica, anti-helmíntica, citotóxica, inseticida e anti-leishmania comprovadas em estudos *in vitro*. Há interesse no estudo farmacológico *in vitro* dessas substâncias naturais e de seus derivados semi-sintéticos, porém a quantidade obtida nos processos de extração tem se mostrado um fator limitante à continuação desses estudos. No presente trabalho, descrevemos um método para obtenção de gramas desses quassinóides cuja purificação depende apenas de cristalização fracionada.

PALAVRAS CHAVE: Caferana, Simaroubaceae, Quassinóide, Recristalização.

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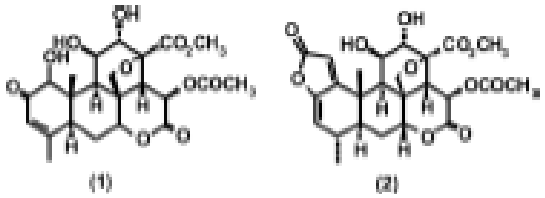
Isobrucein B (1) (Kupchan *et al.*, 1975) and neosergeolide (2) (Schpector *et al.*, 1994) are bioactive secondary metabolites belonging to the class of highly oxygenated terpenoids known as quassinoids, a class which is exclusively found in the Simaroubaceae plant family (Figure 1). Their isolation on small scales has been described previously from the stems and roots of *Picrolemma sprucei* (synonym *P. pseudocoffea* Ducke) (Moretti *et al.*, 1982), one of several plant species known in the Brazilian Amazon as "caferana" (Silva *et al.*, 1977). Other species known as caferana are *Tachia guyanensis* Aubl. (Gentianaceae) and *Quiina rhytidopus* Tul. (Quiinaceae) (Corrêa, 1926; Silva *et al.*, 1977). Compound 1 was also obtained from the leaf extract of *P. sprucei* (Polonsky *et al.*, 1984). The antileukemic, antifeedant, antimalarial (Fandeur *et al.*, 1985; Andrade-Neto *et al.*, 2007), leishmanicidal and anthelmintic (Nunomura *et al.*, 2006) activities of these quassinoids have been reviewed recently (Amorim and Pohlit, 2006) and a study on the cytotoxic, larvicidal, antimalarial and hemolytic activity of 1 and 2 and two semi-synthetic derivatives has been recently published (Silva *et al.*, 2009).

Despite more than 25 years of interest on the part of chemists and pharmacologists in quassinoids from *P. sprucei*, methods for obtaining them are still both resource and time-consuming. Moreover, non-sustainable extraction directly from the Amazon forest is the available method for obtaining *P. sprucei* at present (Amorim and Pohlit, 2006). This fact limits the potential usefulness of this Amazonian medicinal plant and the quassinoids obtained from it. Studies are underway to develop sustainable production methods for this plant and its tissues (Barros and Pohlit, unpublished work). In this report, we describe a method for obtaining both 1 and 2 on gram-scales without the need for preparative chromatographic methods and using fractional recrystallization in the purification step.

Collection was performed in Silves Municipality, in Amazonas State, Brazil, in April, 2003. Voucher specimens have been previously deposited at the UFAM Herbarium (Silva 5729 & 5730). Identification was performed by Dr. Wayt Thomas as *Picrolemma sprucei* Hook. f.(Wayt Thomas, personal comm.). Roots and stems were cut into small pieces while fresh and allowed to dry in the shade and were then ground.



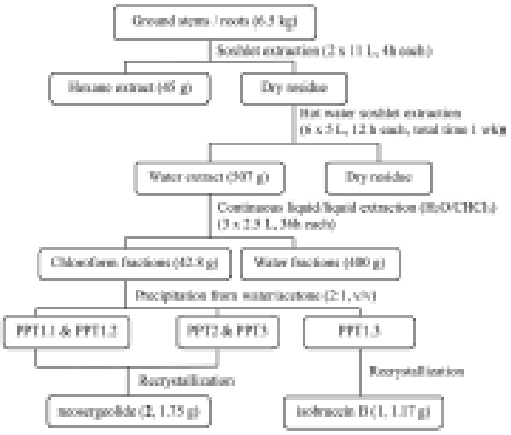
(1) (2)

Figure 1 - Quassinoids obtained from *Picrolemma sprucei*.

In a pilot-scale, stainless-steel Soxhlet extractor, powdered stems and roots were degreased by continuous extraction with hexanes. The solvent was evaporated from the marc which was then exhaustively extracted in the same apparatus with distilled hot water. The water extracts were combined and concentrated using rotatory evaporation *in vacuo* with a 50-55°C bath. The resulting concentrated water extract was re-suspended in 700 mL of water and continually extracted with chloroform. The three concentrated chloroform extracts (totaling 42.8 g, after total evaporation) were each separately dissolved in a minimum of hot water and acetone (2:1, v/v) (Silva, 2006). Crystallization occurred upon cooling of the resulting solutions. The first chloroform extract (CF1) yielded three crops of crystals (PPT1.1, PPT1.2 and PPT1.3) and a filtrate. The second and third chloroform extracts (CF2 and CF3) yielded one crop each (PPT2 and PPT3, respectively). After recrystallization, PPT1.1, PPT1.2, PPT2 and PPT3 yielded a total of 1.75 g (0.027 %) of pure 2. Recrystallizations of PPT1.3 yielded 1.17 g (0.018 %) of pure 1 (Scheme 1). Identification was performed by comparison of physical and spectral data with literature data (Moretti *et al.*, 1982; Vieira *et al.*, 2000). The above isolation and purification procedures were monitored by analyzing extracts, fractions and substances by normal-phase thin-layer chromatography (TLC) using ether : isopropyl alcohol (9:1) as mobile-phase. TLC plates were conveniently developed by contact with iodine vapor and illumination with UV light (254nm). The R_f values for 1 and 2 were 0.6 and 0.4, respectively under these conditions. The melting points (m.p.) obtained for 1 and 2 were 258-260.1 °C and 202-204.1 °C, respectively.

Moretti *et al.* (1982) used degreasing and exhaustive, serial hot water infusions which are a lengthy and tedious procedure, especially on a pilot scale. In our adaptation, the use of continuous liquid-solid extraction for these tandem steps provided quassinoid-rich, partially concentrated water extracts. Whereas in Moretti *et al.* (1982) the chloroform extract was chromatographed to yield 1 and 2 on milligram scales, our method obviates chromatography altogether. The key recrystallization step removes polar, water soluble substances present in the chloroform extract and provides for differential recrystallization of 1 and 2.

This method should aid researchers interested in obtaining 1 and 2 for pharmacological studies. Cold and room temperature extraction with non-chlorinated solvents, followed by recrystallization, are under study to further develop multi-gram isolation of 1 and 2.



Scheme 1 - Flowchart of the gram-scale process of neosergeolide and isobrucein B extraction and purification from *P. sprucei*.

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CAPÍTULO 4: Atividade biológica da neosergeolida e isobruceína B isolados da planta medicinal Amazônica *Picrolemma sprucei* (Simaroubaceae) e dois derivados semi-sintéticos

Biological activity of neosergeolide and isobrucein B (and two semi-synthetic derivatives) isolated from the Amazonian medicinal plant *Picrolemma sprucei* (Simaroubaceae)

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In the present study, in vitro techniques were used to investigate a range of biological activities of known natural quassinoids isobrucein B (1) and neosergeolide (2), known semi-synthetic derivative 1,12-diacetylisobrucein B (3), and a new semi-synthetic derivative, 12-acetylneosergeolide (4). These compounds were evaluated for general toxicity toward the brine shrimp species Artemia franciscana, *cytotoxicity toward human tumour cells, larvicidal activity toward the dengue fever mosquito vector* Aedes aegypti, *haemolytic activity in mouse erythrocytes and antimalarial activity against the human malaria parasite* Plasmodium falciparum. *Compounds 1 and 2 exhibited the greatest cytotoxicity against all the tumor cells tested (IC_{50} = 5-27 µg/L) and against multidrug-resistant* P. falciparum *K1 strain (IC_{50} = 1.0-4.0 µg/L) and 3 was only cytotoxic toward the leukaemia HL-60 strain (IC_{50} = 11.8 µg/L). Quassinoids 1 and 2 (LC_{50} = 3.2-4.4 mg/L) displayed greater lethality than derivative 4 (LC_{50} = 75.0 mg/L) toward* A. aegypti *larvae, while derivative 3 was inactive. These results suggest a novel application for these natural quassinoids as larvicides. The toxicity toward* A. franciscana *could be correlated with the activity in several biological models, a finding that is in agreement with the literature. Importantly, none of the studied compounds exhibited in vitro haemolytic activity, suggesting specificity of the observed cytotoxic effects. This study reveals the biological potential of quassinoids 1 and 2 and to a lesser extent their semi-synthetic derivatives for their in vitro antimalarial and cytotoxic activities.*

Key words: neosergeolide - isobrucein B - 12-acetylneosergeolide - 1,12-diacetylisobrucein B - cytotoxicity - antimalarial - larvicide

The global malaria situation has worsened in the last two decades with an estimated 350-500 million new cases and more than one million annual deaths attributed to this disease, 90% of which have been recorded in Africa (WHO 2005, CDC 2008). Global efforts toward controlling malaria are greatly challenged by the increasing spread of antimalarial drug resistance and the use of ineffective antimalarials is considered partially responsible for the difficulties in reducing malaria morbidity and mortality (Vastergaard & Ringwald 2007). New drugs are therefore needed to treat malaria. Interest in plants as sources of new antimalarials has been stimulated by the isolation of artemisinin from *Artemisia annua*. This compound is active against drug-resistant *Plasmodium falciparum*, the most lethal human malaria parasite species (van Agtmael et al. 1999). The Brazilian Amazon, where human malaria is endemic, is one of the richest sources of plants with potential pharmacological activity. Plants are frequently used to treat the disease as infusions or as additives in alcoholic beverages (Milliken 1997). *Picrolemma pseudocoffea* Ducke (pseudonym for *Picrolemma sprucei* Hook. f.) is one of a number of plant species known in the Brazilian Amazon as *cafezana* and is commonly used for the treatment of malaria throughout the Amazon region (Grenand et al. 1987, Duke & Vasquez 1994, Milliken 1997).

In the investigation of the biological activity of plant extracts and natural products isolated from plants, the brine shrimp assay is a valuable tool for establishing general toxicity and cytotoxicity parameters. This assay consists of exposing brine shrimp larvae to plant extract in saline solution and larval mortality is evaluated after one day. A very positive correlation between the lethality to brine shrimp and cytotoxicity has been established

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by researchers working on the development of new anticancer drugs from plants at the National Cancer Institute in the United States (Anderson et al. 1991). This correlation is considered so good that lethality toward brine shrimp is recommended by these authors as an effective pre-screen for existing in vitro cytotoxicity and antitumor assays (Anderson et al. 1991, Quignard et al. 2003).

Invasive mosquitoes are economic and sanitary concerns, especially in Europe and America (Boyer et al. 2006). Among the mosquito vectors of most concern is *Aedes aegypti* (Diptera: Culicidae) which originated in Africa, has spread around the world and is a vector of yellow and hemorrhagic dengue fever. The present resurgence of these diseases is due to the higher number of breeding places for these mosquitoes in today's throwaway society (Kiran et al. 2006). Additionally, temephos, a synthetic organophosphate compound widely used as insecticide and larvicide in Brazil presents related resistance cases and therefore a loss of overall efficacy (Lima et al. 2006) which is compounded in tropical countries having high annual rainfalls by the need for as little as 1-2 mL of clean water for this species to carry out the aquatic portion of its life cycle, from egg through larval phase to emergent imago (adult). For these reasons and despite significant advances in techniques used for its control during recent decades, *A. aegypti* and other mosquitoes continue to pose serious public health problems (Guzmão et al. 2002). Importantly, co-evolution has equipped plants with a plethora of chemical defences against insect predators. As such, mankind has used plants or plant extracts to control insects since ancient times (Balandrin 1985, Sukumar et al. 1991). In previous work, we have shown the larvicidal activity against *A. aegypti* of *P. sprucei* stem and root methanol extracts (Pohlit et al. 2004).

Quassinoid is the name given to any of a number of bitter substances found exclusively in the Simaroubaceae family (Polonsky 1973). Chemically, quassinoids are degraded triterpene compounds which are frequently highly oxygenated. Many quassinoids exhibit a wide range of biological activities in vitro and/or in vivo, including antitumor, antimalarial, antiviral, anti-inflammatory, antifeedant, insecticidal, amoebicidal, antiulcer and herbicidal activities. Bruceantin, simalikalactone D, quassin, brusatol and glaucarubinone are some of the most well-studied quassinoids and exhibit a wide range of biological activities (Guo et al. 2005).

Quassinoid compounds isobrucein B (1) and neosergeolide (2) have been isolated previously from the stems and roots of *P. sprucei* (Moretti et al. 1982, Schpector et al. 1994) and isobrucein B was also obtained from the leaf extract of this same plant species (supplementary data) (Polonsky et al. 1984). Our group has recently demonstrated the important antimalarial activity of 2 (Andrade-Neto et al. 2007), the antihelminthic activity of 1 and 2 (Nunomura et al. 2006) and has written a review in which the noteworthy antileukemic, antifeedant and leishmanicidal activities of 1 were discussed (Amorim & Pohlit 2006). Kupchan et al. (1975) prepared the semi-synthetic quassinoid 1,12-diacetylisobrucein B (3) by acetylation of 1, but no evaluation of its biological activity has been published to date. In general, semi-synthetic modification of natural quassinoids is thought to be a useful tool for the discovery of new therapeutic drugs and quassinoid derivatives with other potential uses and could lead to derivatives with increased therapeutic indices, including significantly increased pharmacological activity and/or decreased toxicity.

Herein, in vitro methods are used to investigate the general toxicity in the brine shrimp assay towards *Artemia franciscana*, the cytotoxicity toward human tumor cells, the larvicidal activity in *A. aegypti*, and the haemolytic and in vitro antimalarial activity against *P. falciparum* of known isolated quassinoids (1 and 2) and semi-synthetic quassinoid derivative 3, as well as a new semi-synthetic derivative, 12-acetylneosergeolide (4).

MATERIALS AND METHODS

Plant materials - Collection was performed in Silves, state of Amazonas (AM), Brazil, in April 2003. Voucher specimens have been deposited at the Universidade Federal do Amazonas Herbarium (Silva 5729 & 5730). Identification was performed by Dr. Wayt Thomas as *P. sprucei* (Wayt Thomas, personal communication).

Isolation and identification of quassinoids - The procedure used for the isolation of quassinoids isobrucein B (1) and neosergeolide (2) from the roots and stems of *P. sprucei* has been described in a previous publication (Andrade Neto et al. 2007).

Spectrometric measurements - Nuclear magnetic resonance (NMR) spectra were obtained on a Varian Unity Inova model, using VnmrJ software. Positive ion electrospray ionization accurate mass spectra [(+)-ESI-MS] were obtained on a Bruker-Daltronics UltrOTof apparatus using MeOH/H_2O/HCO_2H as an infusion solvent system. Melting points were determined on a Marconi MA-381 apparatus and are uncorrected. Fourier-transform infrared spectra were obtained on a BOMEM FTLA-2000-104 and UV spectra were obtained on a Femto 800 XI apparatus.

1,12-diacetylisobrucein B - Treatment of compound 1 (50.6 mg, 105.4 µmol) with pyridine (3.3 mL) and acetic anhydride (3.3 mL) in accordance with Kupchan et al. (1975) yielded 1,12-diacetyl derivative 3 (50.7 mg, 85.2%); mp 240-245°C [264-267°C (Kupchan et al. 1975)]; IR (KBr) 3447 (O-H), 2919-2950 (C-H), 1734-1750 (C = O), 1647 (C-2, C = O) cm^{-1} [(KBr) 3534, 1745, 1681, 1370, 1230, 1036 cm^{-1} (Kupchan et al. 1975)]; UV_{max} (MeOH) 241.0 nm. [UV_{max} (EtOH) 238 nm (Kupchan et al. 1975)]; ^1H NMR (500 MHz, $CDCl_3$) δ 6.06 (s, H-3), 5.38 (s, H-1), 5.26 (s, H-12), 4.79 (d, H-30a, *J* = 8.3), 4.74 (s, H-7), 4.10 (d, H-11, *J* = 4.1), 3.79 (s, H-5'), 3.77 (d, H-30a, *J* = 8.3), 3.25 (d, H-14, *J* = 11.6), 3.03 (d, H-5, J = 13.0), 2.42 (ddd, H-6a, J = 13, 2.0, 2.0), 2.23 (s, H-9') 2.18-2.25 (m, H-9), 2.10 (s, H-2'), 2.01 (s, H-7'), 1.94 (s, H-29), 1.83 (ddd, H-6a, J = 13.0, 13.0, 2.0), 1.39 (s, H-19) [(100 MHz, $CDCl_3$) δ 5.95 (br s, H-3), 5.15 (d, H-15, *J* = 15), 3.69 (s, H-5'), 2.16 (s, H-9'), 2.03 (s, H-2'), 1.95 (s, H-7'), 1.88 (s, H-29), 1.14 (s, H-19) Kupchan et al. 1975)]; ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.57 (C-2), 170.34 (C-8'), 169.41 (C-18), 168.85 (C-1'), 166.64 (C-16), 159.96 (C-4), 126.44 (C-3), 83.84 (C-1), 82.70 (C-7), 80.36 (C-13), 74.68 (C-

12), 73.92 (C-30), 71.23 (C-11), 53.10 (C-5'), 46.13 (C-10), 45.51 (C-8), 43.5 (C-5), 42.52 (C-9), 28.04 (C-6), 22.57 (C-29), 21.04 (C-9'), 20.93 (C-7'), 20.50 (C-2'), 12.24 (C-19); ESI-tof-MS *m/z* 587.1775 $[M+Na]^+$ (exact mass for $C_{29}H_{32}O_{12}Na^+$ 587.1735) [EI-MS *m/z* 564 (M^+), 522, 504, 489, 135, 95, 91, 60, 43 (Kupchan et al. 1975)].

12-acetylneosergeolide - Neosergeolide (2, 16.4 mg, 32.5 µmol), pyridine (1 mL), and acetic anhydride (1 mL) were magnetically stirred for four days at rt. After complete evaporation, the residue was dissolved in methanol (1 mL) resulting in the formation of a precipitate. The precipitate was dissolved in methylene chloride-methanol (1:1) and then submitted to preparative thin-layer chromatography (2% isopropyl alcohol in methylene chloride) which resulted in the isolation of 12-acetylneosergeolide as needles (4, 15.1 mg, 85.1%); (supplementary data) mp 214.6°C (decomp); IR (KBr) 3535 (O-H), 2983-2971 (C-H), 1753 (C-18, C=O), 1679 (C-16, C=O) 1586 (C1'-4, C=O), 1209-1238 (C-O) cm^{-1}; UV_{max} (MeOH) 279.0 nm; ^1H NMR ($CDCl_3$ + CD_3OD) δ 5.96 (s, H-4'), 5.55 (t, H-3, *J* = 2.8), 5.11 (s, H-12), 4.65 (d, H-30s, *J* = 8.3), 4.57 (s, H-7), 4.19 (d, H-11, *J* = 3.0), 3.63 (d, H-30a, *J* = 8.3), 3.60 (s, H-5'), 3.09 (dl, H-14, *J* = 12.5), 2.20-2.24 (m, H-4), 2.05-2.10 (m, H-9 + H-6e), 1.90 (s, H-2'), 1.82 (s, H-7'), 1.50-1.65 (m, H-5 + H-6a) 1.39 (s, H-19), 1.00 (s, H-29); ^{13}C NMR ($CDCl_3$) δ 172.3 (C-6'), 170.9 (C-3'), 169.8 (C-18), 169.2 (C-1'), 167.2 (C-16), 162.5 (C-1), 148.5 (C-2), 116.3 (C-3), 112.9 (C-4'), 83.4 (C-7), 79.9 (C-13), 75.3 (C-12), 73.5 (C-30), 70.7 (C-11), 52.7 (C-5'), 49.1 (C-14), 46.0 (C-8), 44.9 (C-5), 41.8 (C-10), 39.3 (C-9), 30.7 (C-4), 28.4 (C-6), 20.5 (C-7'), 20.0 (C-2'), 19.2 (C-29), 17.8 (C-19); ESI-tof-MS *m/z* 547.1810 $[M+H]^+$ (calculated exact mass for $C_{29}H_{31}O_{12}^+$ 547.1810).

Brine shrimp assay - The lethality of quassinoids toward nauplii (larvae) of the brine shrimp species *A. franciscana* Kellogg was evaluated according to established procedures (Meyer et al. 1982, Anderson et al. 1991, Mongelli et al. 1996, Parra et al. 2001, Quignard et al. 2003). Quassinoids and derivatives were separately dissolved and diluted in DMSO to provided sample solutions having 10 concentrations ranging from 1.000.0-0.250.25 µg/mL. The co-solvent DMSO had final well concentrations of ≤ 1% in all experiments and controls. For the brine shrimp assay, wells of 24-well microtitre plates were pre-filled with 0.8 mL saline solution. Next, 10 two-day old nauplii (2nd instar larvae) in a minimum volume of saline solution (ca. 0.2 mL), 10 µL of each quassinoid dissolved in DMSO and a stock saline solution were added, which provided a final volume of 1.0 mL in each well (Mosmann 1983). Each compound was tested in three independent assays, each in triplicate. The plates were then allowed to stand in the absence of direct light (to avoid possible false positive results due to the generation of phototoxic compounds) at rt and mortality was evaluated after 24 h. The half-maximal inhibitory (IC_{50}) responses, as compared to the drug-free controls, were estimated by Probit analysis using Microsoft Excel® software.

MTT assay - Quassinoids and derivatives were tested for cytotoxicity in four human tumour cell lines: SF295 (glioblastoma), MDA-MBA345 (melanoma), HCT-8 (colon) and HL-60 (leukemia) (Children's Mercy Hospital, Kansas City, MO, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin and incubated at 37°C under a 5% CO_2 atmosphere. Cells were then plated in 96-well plates (10^5 cells/well for adherent cells or 0.5 x 10^5 cells/well for suspended cells in 100 µL of medium). After 24 h, compounds (25.0 to 0.001 µg/mL in 17 dilutions) dissolved in 1% DMSO were added to each well and incubated for 72 h. Doxorubicin (0.01-0.38 µg/mL) was used as positive control. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide to a purple formazan product (Mosmann 1983). The drug effect was quantified as the percentage of the absorbance of reduced dye at 595 nm in relation to control wells. The IC_{50} responses as compared to the drug-free controls were estimated by interpolation using Prism4® software.

Larvicidal activity in A. aegypti - Larvicidal activity of quassinoids toward *A. aegypti* was evaluated using the method described in Pohlit et al. (2004). *A. aegypti* larvae were obtained by incubation of eggs from a laboratory colony (originated from an AM field strain) at Coordenação de Pesquisas em Ciências da Saúde, Instituto Nacional de Pesquisas da Amazônia, in tap water. Quassinoids and derivatives (0.1 to 100 µg/mL in 11 dilutions) were dissolved in DMSO, which was used as the co-solvent at final well concentrations of ≤ 1% in all experiments and controls. Temephos (0.01-0.38 µg/mL) was used as positive control. Negative controls contained 1% DMSO instead of sample solution. Each experiment and controls were performed in three independent assays, each in triplicate, and compared with controls after 24 h and 48 h at 26-27°C. The IC_{50} responses as compared to the drug-free controls were estimated by Probit analysis using Microsoft Excel® software.

Parasite culture and in vitro antimalarial tests - Chloroquine, pyrimethamine and cycloguanil resistant *P. falciparum* strain K1 was acquired from MR4 (Malaria Research and Reference Reagent Resource Centre, Manassas, Virginia, USA) and was used in all in vitro tests. Parasites were maintained in continuous culture in A+ human erythrocytes using RPMI medium supplemented with 10% human serum, as described by Trager and Jensen (1976). The antiparasitic effect of the compounds was measured by growth inhibition percentages as described by Carvalho and Krettli (1991). Trophozoite-stages in sorbitol-synchronized blood (Lambros & Vanderberg 1979) were cultured at 1-2% parasitaemia and 2.5% haematocrit and then incubated with compounds (maximum 1 mg/mL in serial dilutions) diluted with a 0.02% final concentration of DMSO in the culture medium (RPMI 1640) for a total of 48 h at 37°C. Positive controls contained reference antimalarial drugs (chloroquine, quinine and artemisinin) in standard concentrations (Rieckmann et al. 1978, WHO 2001) and were used for each experiment. The stock solutions were further diluted with complete medium (RPMI 1640 plus

10% human serum) to each of the concentrations used (100-0.0001 µg/mL in 7 dilutions). The IC_{50} responses as compared to the drug-free controls were estimated by interpolation using Microcal Origin® software. Each duplicate experiment was repeated three times and blood smears were read blind (Andrade-Neto et al. 2007).

Haemolytic assay - This test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the method described by Costa-Lotufo et al. (2002). Compounds were tested at concentrations ranging from 1.5-200 µg/mL. 0.1% Triton X-100 (in 0.85% saline) was used as a positive control. After incubation at rt for 1 h and centrifugation, the supernatant was removed and the liberated haemoglobin was measured spectrophotometrically as the absorbance at 540 nm.

Statistical analysis - Data are presented as mean value ± SEM. The IC_{50} or EC_{50} values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). LC_{50} values for the brine shrimp assay were obtained from 24 h larvae counts using the probit analysis method described by Finney (1971).

RESULTS AND DISCUSSION

Known and new quassinoid derivatives 3 and 4, respectively, were prepared using simple acetylation-purification procedures and the ESI-MS, IR and NMR spectra were consistent with the proposed structures. Importantly, spectroscopic evidence supports the regiochemical outcome of the acetylation, which could, *a priori*, have occurred at either or both the C-11 or C-12 hydroxyl group. NMR-based evidence suggests that the C-12 hydroxyl group undergoes acetylation (together with the C-1 hydroxyl in the case of isobrucein B acetylation) preferentially over the C-11 hydroxyl group. Namely, the signal at δ 5.11 in the ^{1}H-NMR spectrum of 4 was assigned to H-12. In the Heteronuclear Single Quantum Coherence spectrum, this signal had a ^{13}C-^{1}H correlation with δ 75.31 (C-12). The H-12 signal of 4 was shifted approximately 1 ppm to lower field relative to H-12 (δ 4.23) of the precursor 2. This downfield shift is evidence for the deshielding caused by the electronegative acetate group on C-12 in derivative 4. Furthermore, the assignment of H-12 (δ 5.11) is supported by long-range ^{13}C-^{1}H correlations with C-13 (δ 79.9) and C-18 (δ 169.8) in the Heteronuclear Multiple Bond Correlation spectrum as well as correlation with neighbouring H-11 (δ 4.19) in the ^{1}H-^{1}H Correlation Spectroscopy spectrum of 4.

The brine shrimp assay is a simple, inexpensive method which allows for the evaluation of general toxicity and is considered a preliminary assay in the study of extracts and secondary metabolites for cytotoxic and antitumor activity. LC_{50} values in the brine shrimp assay correlate well with other in vitro tests such as those for antitumor activity, cytotoxicity (Meyer et al. 1982, Munro et al. 1987, Siqueira et al. 2001) and antineoplastic activity (Badaway & Kappe 1997) and also for in vivo toxicity (LD_{50}) of extracts administered by gavage in Swiss albino mice (Parra et al. 2001) and pesticidal (Fatope et al. 1993), larvicidal, fungicidal, molluscicidal (Cepleanu et al. 1994), antimalarial (Perez et al. 1997), insecticidal (Oberlies et al. 1998) and antifeedant activities (Labbe et al. 1993). Quassinoids 1 and 2 were considered very toxic toward *A. franciscana*, while 3 showed low toxicity (Fig. 1, Table). The same was observed for the MTT cytotoxicity tests, where 1 and 2 exhibited IC_{50} values ranging from 0.005-0.027 µg/mL for all tumor cell lines, while 3 and 4 exhibited IC_{50} varying from 0.51 to > 25 µg/mL (Fig. 2, Table).

In preliminary work, it was shown that at a concentration of 500 µg/mL, *P. sprucei* stem and root methanol extracts caused 57% and 74% mortality in *A. aegypti* larvae, respectively (Pohlit et al. 2004). In the present study, since quassinoids 1 and 2 are present in methanol and other polar extracts, the potential larvicidal activity of 1 and 2, as well as their acetylated derivatives 3 and 4, respectively, were investigated. The results showed that 1, 2 and 4 were active against *A. aegypti* third instar larvae (Fig. 3); compound 3 showed no activity at a dose of 100 µg/mL. As such, quassinoids 1 and 2 present in *P. sprucei* could be partially responsible for the larvicidal activity exhibited by extracts of this plant. The synthetic compound temephos was very lethal toward *A. aegypti* larvae (IC_{50} = 0.025 µg/mL). Quassinoids and their derivatives were less active than temephos, but could be important models for the further development of new larvicides. There is no data on the effectiveness of these compounds in a field setting, nor is it known whether toxicity toward other organisms could be a problem in a field application. Notably, due to reported resistance cases and concerns associated with permanence of organophosphates in the environment over extended periods of time, there is a need to find substitutes for or alternatives to organophosphate insecticides (Lima et al. 2006).

Significant inhibition of the human malaria parasite *P. falciparum* in vitro was exhibited by quassinoids 1, 2 and derivative 4. Ranked on a molar scale, the order of increasing inhibition of parasites was 3 < 4 < 2 < 1 (IC_{50} = 34.91, 0.216, 0.008 and 0.002 µM, respectively) (Fig. 4). Chloroquine, quinine and artemisinin displayed IC_{50} values of 0.89, 0.012 and 0.002 µM, respectively. As such, neosergeolide (2) and isobrucein B (1) presented the highest activity, in the same range of activity as the clinically important drug standards that were used as controls. Despite moderate parasite inhibition, compound 4 was four times more active than chloroquine, while 3

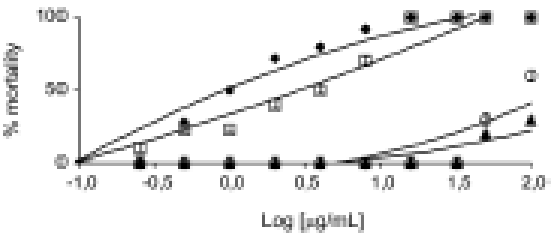

Fig. 1: toxic activity of natural and semi-synthetic quassinoids obtained from *Picrolemma sprucei* against the brine shrimp *Artemia franciscana*. ●: isobrucein B (1); ○: neosergeolide (2); ▲: 1,12-diacetylisobrucein B (3); ◇: 12-acetylneosergeolide (4).

TABLE

Biological activity of isolated quassinoids *Picrolemma sprucei* and their semi-synthetic derivatives

	Biological assay						
	Artemia franciscana	*Aedes aegypti*	Citotoxicity in tumor cell lines IC_{50} (μg/mL)				*Plasmodium falciparum*
Compound	LC_{50} (μg/mL)	LC_{50} (μg/mL)	SF295	MDA-MBA345	HCT-8	HL-60	IC_{50} (μg/mL)
1	1.3 ± 0.1	3.2 ± 0.6	0.027 ± 0.01	0.027 ± 0.02	0.024 ± 0.03	0.027 ± 0.01	0.001 ± 0.002
2	0.9 ± 0.1	4.4 ± 0.2	0.018 ± 0.01	0.008 ± 0.02	0.005 ± 0.01	0.007 ± 0.01	0.004 ± 0.0025
3	nd[a]	nd[a]		nd[b]	nd[a]	11.77 ± 0.02	20.5 ± 10.6
4	147 ± 9.0	75.0 ± 7.5	0.51 ± 0.02	0.76 ± 0.01	0.61 ± 0.01	0.51 ± 0.02	0.118 ± 0.09
Doxorubicin			0.25 ± 0.01	0.47 ± 0.02	0.04 ± 0.01	0.02 ± 0.01	
Quinine							0.06 ± 0.03
Chloroquine							0.082 ± 0.052
Artemisinin							0.0008 ± 0.0005

a: > 100 μg/mL; *b*: > 25 μg/mL; nd: not determined. Values are means ± SEM.

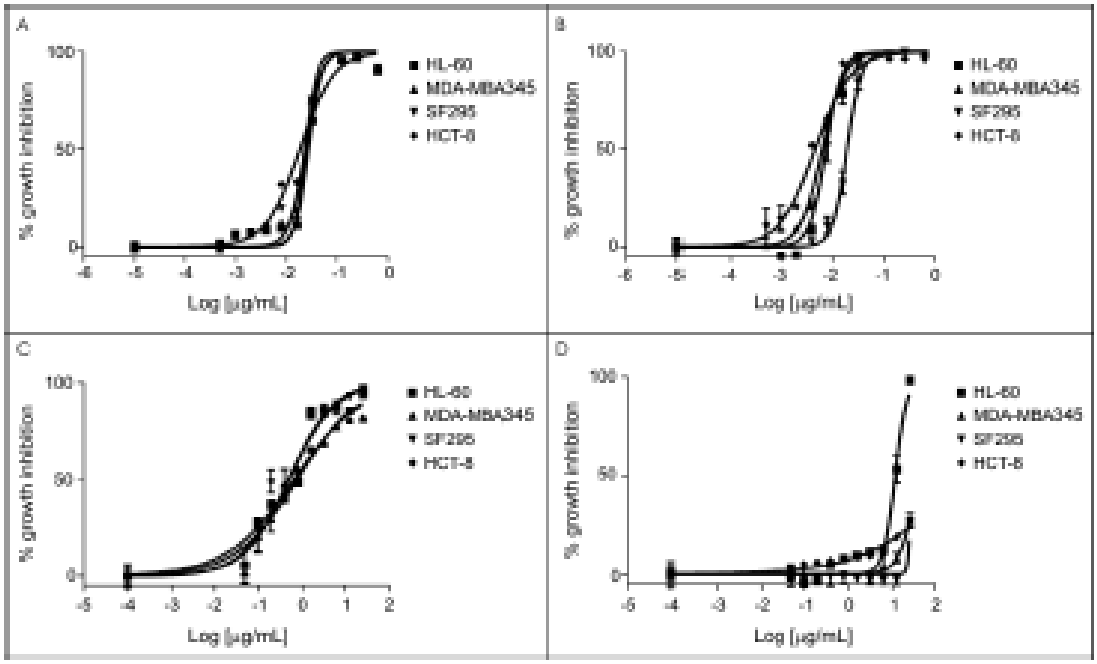

Fig. 2: cytotoxic activity of natural quassinoids obtained from *Picrolemma sprucei* and semi-synthetic derivatives. A: isobrucein B (1); B: neosergeolide (2); C: 1,12-diacetylisobrucein B (3); D: 12-acetylneosergeolide (4). Human tumor cell lines: HCT-8: colon; HL-60: leukemia; MDA-MBA345: melanoma; SF295: glioblastoma.

showed very weak activity relative to natural quassinoids and positive controls. The in vitro sensitivity of the *P. falciparum* strain toward the compounds tested was similar and reproducible in assays in duplicate on separate occasions. Some quassinoid structural requirements, such as an α,β-unsaturated ketone in the A ring, an epoxymethylene bridge on the C ring and an ester functionality at C-15 are considered important for the antimalarial activity presented by quassinoids (Okano et al. 1995) and are in general present in the compounds under study.

An ethnopharmacological study in French Guyana indicated that the *P. sprucei* root, stem and bark alcohol extract is used in local traditional medicine as a curative treatment for malaria in association with *Geissospermum* spp. (Apocynaceae) and *Quassia amara* (Simaroubaceae) or modern drugs (Vigneron et al. 2005). In a subsequent study, Bertani et al. (2005) demonstrated that *P. sprucei* water extract can inhibit hemozoin formation. In vitro assays demonstrated the antimalarial activity of this extract against the chloroquine-resistant

P. falciparum strain W2. In the present study, we have demonstrated that both quassinoids 1 and 2 are isolated from infusions of *P. sprucei* in the laboratory procedure described and it is reasonable to assume that they are at least partially responsible for the antimalarial activity reported previously for *P. sprucei* water extracts by Vigneron et al. (2005) and Bertani et al. (2005).

The erythrocytic membrane is a delicate structure that can be significantly altered by drug interactions (Aki & Yamamoto 1991). Several studies indicate that certain compounds isolated from plants such as polyphenols, glycosides, saponins and triterpenoids may cause changes in the membranes of red blood cells and subsequently produce haemolysis (Ng et al. 1986, Bader et al. 1996, Grinberg et al. 1997, Zhang et al. 1997). The mechanical stability of the erythrocyte membrane is a good indicator of in vitro insults in programs for cytotoxicity screening, since its structural dynamics favour interactions with drugs and changes that may be assessed by protein electrophoresis (Sharma & Sharma 2001). Erythrocytes also provide a simple model for the study of the protective or toxic effects of substances or situations associated with oxidative stress (Aparicio et al. 2005, Lexis et al. 2006, Muñoz-Castañeda et al. 2006). Our results suggest that the observed high cytotoxicity of quassinoids towards tumor cell lines is not due to oxidative stress or damage to the cell membrane, since none of the quassinoids or their derivatives were observed to lyse mouse erythrocytes at concentrations below 200 µg/mL.

Natural quassinoids 1 and 2 display higher activities than their acetylated derivatives for all biological tests performed (except haemolytic activity, where as was stated above, no activity was observed for any of these compounds). This is evidence that the presence of a hydroxyl group at C-12 may be important for the cytotoxic, larvicidal and antimalarial activities observed in the present study. The enone group in ring A of substances 1 and 3 likely acts as a Michael acceptor for biological

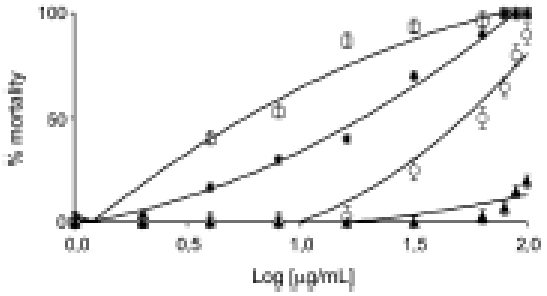

Fig. 3: larvicidal activity of natural and semi-synthetic quassinoids obtained from *Picrolemma sprucei* against *Aedes aegypti* larvae. ♦: isobrucein B (1); ○: neosergeolide (2); ▲: 1,12-diacetylisobrucein B (3); ◇: 12-acetylneosergeolide (4).

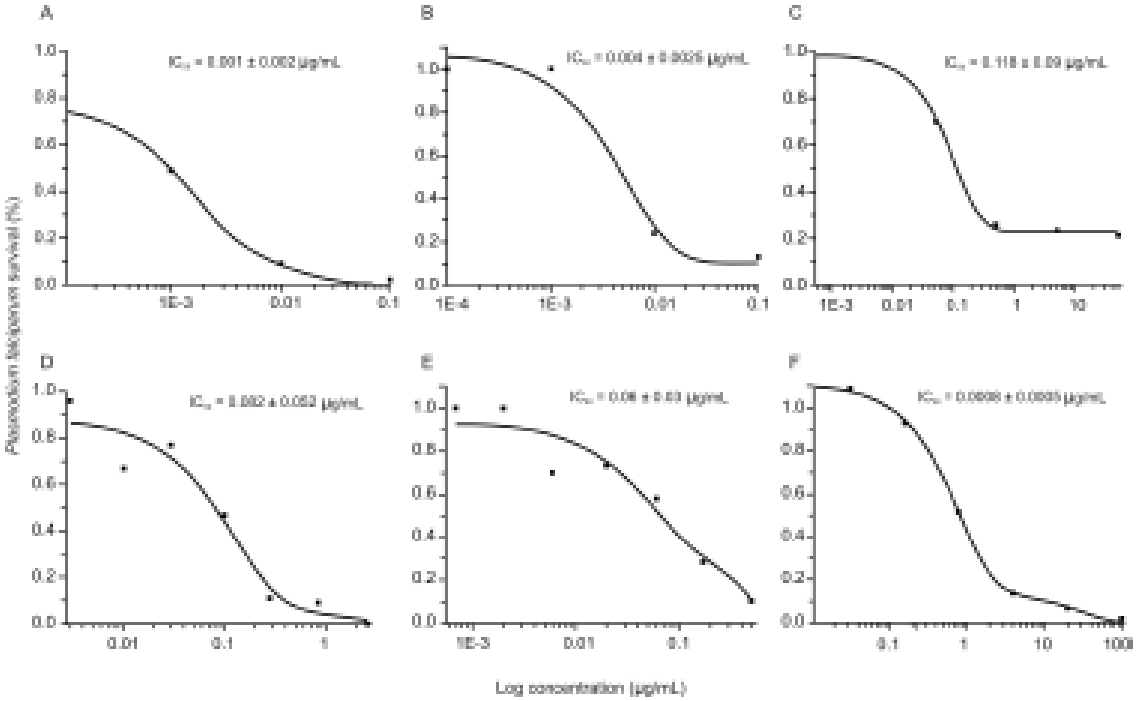

Fig. 4: dose-response curve from *Plasmodium falciparum* K1 parasite survival (chloroquine resistant) in the presence of various concentrations of quassinoid compounds and antimalarial drugs tested. A: isobrucein B (1); B: neosergeolide (2); C: di-acetylisobrucein B (3); D: chloroquine; E: quinine; F: artemisinin. Values are means ± SEM in representative experiment in duplicate. Significant differences in relation to controls by Mann-Whitney *U*-test with $p < 0.05$.

nucleophiles (Kupchan et al. 1975). This is strongly supported by the significant increase in biological activity associated with the presence of a free hydroxyl group at C-1 and/or C-3 in quassinoids (as 1 has a free hydroxyl group at C-1, while its inactive derivative 3 does not). This is presumably due to intramolecular hydrogen bonding between these hydroxyl groups and the enone oxygen atom, further activating the enone moiety towards nucleophilic attack (Guo et al. 2005). Additionally, diminished cytotoxic activity (28, 72, 95 and 122 times less against SF295, HL-60, MDA-MBA345 and HCT-8, respectively) of 4 (no free hydroxyl group at C-12) in comparison to 2 (free hydroxyl group at C-12) is evidence that the hydroxyl group at C-12 is crucial for the observed cytotoxicity (Table). Similar data are available for a number of quassinoids bearing A-D ring structures analogous to 1 and 2 and quantitative structure-activity analyses have attributed this cytotoxic activity to the presence of carbonyl and hydroxyl groups on the A ring, an epoxymethylene bridge between C-8–C-13 and a side-chain containing an ester (oxycarbonyl) group at C-15 (Okano et al. 1995).

Quassinoids 1 and 2 from *P. sprucei* display important in vitro cytotoxic, antiplasmodial and larvicidal activities. The antiplasmodial activity described above, especially for 1, and anthelmintic activity previously described by us for 1 and 2 lend support to traditional medicinal practice and recent ethnobotanical and ethnopharmacological studies all of which indicate the efficacy of *P. sprucei* (Vigneron et al. 2005, Numomura et al. 2006)

In general, (di)acetylated semi-synthetic quassinoid derivatives 3 and 4 displayed less in vitro cytotoxic, antiplasmodial and larvicidal activity than the natural compounds from which they were derived. The effective larvicidal activity exhibited by quassinoids underscores the need for more specific tests for the possible effects on the peritrophic matrix structure, as antifeedants, and for effects of quassinoids on other larval stages. Tests for insecticide activity in *A. aegypti* imagoes (adults) and mosquitos of the *Anopheles* and other genera, studies of synergism and in vivo antitumor and antimalarial (including artemisinin resistant strains) activities and the mechanism of cytotoxic activity are underway and the results will be reported in due course. Additionally, the preparation of novel semi-synthetic quassinoid derivatives may provide compounds with more adequate physical and medicinal properties (stability, solubility, toxicity, specific action mechanisms) and more detailed information about the structure-activity relationships.

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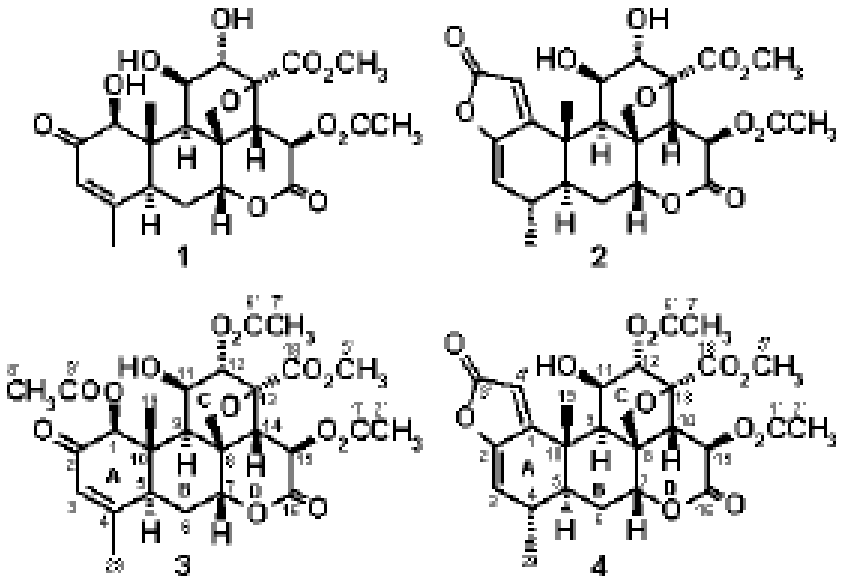

Natural and semi-synthetic quassinoids obtained from *P. sprucei*.

CAPÍTULO 5: Determinação de quassinóides isobruceína B e neosergeolida por LC-ESI-MS em infuses de caules de *Picrolemma sprucei*

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Article

LC-ESI-MS Determination of Quassinoids Isobrucein B and Neosergeolide in *Picrolemma sprucei* Stem Infusions

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Infusões dos caules de *Picrolemma sprucei* (pseudônimo: *P. pseudocoffea*) são principalmente utilizadas como antimaláricos em toda região amazônica. Desta espécie foram isolados os quassinoides isobruceína B e neosergeolida, os quais apresentam atividade antimalárica e citotóxica. Neste estudo, apresentamos o desenvolvimento de uma metodologia analítica por LC-(+)-ESI-MS/MS visando a determinação dos principais quassinoides desta espécie. O método desenvolvido foi empregado para análise de uma formulação artesanal (infusão de 1 g de matéria seca - caules pulverizados de *P. sprucei* em 1 L de água fervente). Padrões previamente isolados de isobruceína B e neosergeolida foram utilizados para a determinação da linearidade na faixa de calibração entre 0,25 to 5 μg mL^{-1} e 0,5 a 10 μg mL^{-1}, respectivamente. Como padrão interno foi utilizada a substância fluoroglucinol na concentração de 4,0 μg mL^{-1}. Ambos os compostos apresentaram boa linearidade, precisão e exatidão e as concentrações de isobruceína B e neosergeolida obtidas nas infusões foram de 60,1 e 774 μg L^{-1}, respectivamente.

Infusions of the stems of *Picrolemma sprucei* (pseudonym: *P. pseudocoffea*) are used in the Amazon regions of Peru, Brazil and French Guiana as antimalarials among other uses. They contain the bitter quassinoids isobrucein B (**1**) and neosergeolide (**2**) that have important antimalarial and toxic properties among others. In this study, an LC-(+)-ESI-MS/MS method was developed and applied to the determination of **1** and **2** in a common remedy prepared by infusing 1 g of dry, powdered stems of *P. sprucei* in 1 L of boiling water. Isolated **1** and **2** were used in calibration ranges of 0.25 to 5 μg mL^{-1} and 0.5 to 10 μg mL^{-1}, respectively, with the internal standard phloroglucinol at 4.0 μg mL^{-1}. Good linearity, precision and accuracy were observed for both compounds. The concentrations of **1** and **2** in the stem infusions were found to be 60.1 and 774 μg L^{-1}, respectively.

Keywords: caferana, cafe lane, *Picrolemma sprucei*, *Picrolemma pseudocoffea*, neosergeolide, isobrucein B, LC-MS/MS

Introduction

One of the classic goals of the Brazilian natural products research community is the search for new biologically active compounds.[1] More recently, new interests include investigations of daily and seasonal rhythms of secondary metabolite biosyntheses and quality control of medicinal plants.[2] *Picrolemma sprucei* Hook. f. (Simaroubaceae) is one of several medicinal plant species which are known in the Brazilian Amazon by the common name caferana (meaning "false-coffee", and giving rise to the often used pseudonym for this plant, *P. pseudocoffea* Ducke). It is widely distributed throughout the Amazon region from Peru where it is popularly known as sacha-café[3] to French Guiana where it is called café lane.[4,5] Root, stem and whole plant infusions of *P. sprucei* are used throughout

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the Amazon region in the treatment of malaria,[6] gastro-intestinal problems and intestinal worms.[3] At higher doses, *P. sprucei* infusions have notable toxic effects and are used to provoke abortions.[7]

Recently, *P. sprucei* has been the subject of several studies whose results in general lend support to its uses in traditional medicine. For example, leaf and stem infusions and other extracts have been shown to possess *in vitro* antihelminthic activity.[8] Also, an ethnopharmacological study in French Guiana showed that alcohol extracts of *P. sprucei* root, stem and bark are used in local traditional medicine as a curative treatment for malaria in association with *Geissospermum* spp. (Apocynaceae) and *Quassia amara* (Simaroubaceae) or modern drugs.[5] In a subsequent study, Bertani *et al.*[9] demonstrated that a *P. sprucei* water extract can inhibit hemozoin formation and *in vitro* assays demonstrated the antimalarial activity of this extract against the chloroquine-resistant *Plasmodium falciparum* strain W2. In the western Brazilian Amazon, dried stems are commonly commercialized in local marketplaces by traditional healers or raizeiros and are typically used in the preparation of infusions for oral ingestion.

Infusions of *P. sprucei* contain the quassinoids isobrucein B (**1**) and neosergeolide (**2**) (Figure 1). These compounds have been isolated previously from the stems and roots of *P. sprucei* on a milligram scale[10] and more recently on a gram-scale.[11] Also, **1** has been isolated from the leaves of this plant.[12] Recently, the antimalarial, antileukemic, antifeedant and leishmanicidal activities of **1** were the subject of a review.[13] Finally, **1** and **2** display significant cytotoxicity to human tumor cell lines, no hemolytic activity to mouse erythrocytes, and moderate larvicidal activity towards *Aedes aegypti* (hemorrhagic dengue fever vector).[14]

Considering the biological activities of *P. sprucei* and of the quassinoids isolated from it, together with the possible occurrence of seasonal and circadian variation, stimulates and justifies the development of analytical methods for the quantification of these active metabolites in infusions used in traditional medicine. Liquid chromatography (LC) coupled to mass spectrometry (LC-MS) has emerged as the most specific analytical method for qualitative and quantitative analyses of natural products.[15] Additionally, advances in the elucidation of electrospray ionization (ESI) processes have allowed for the analysis of metabolites with less competition from low oxidative potential processes through molecular ion dissociation[16] in addition to the well established acid-base and cationizing (anionizing) reactions.[17] For these reasons, LC-ESI-MS predominates in Brazil and around the world as the technique of choice in the analysis of natural products of low-volatility in polar analytes of diverse origins.[1] Given the range of dosages of traditionally used infusions of *P. sprucei*, the toxic effects associated with larger doses, and the ability of ESI coupled systems to analyze natural products, the aim of the present study was to develop a new method for determination of **1** and **2** in infusions of stems of *P. sprucei*.

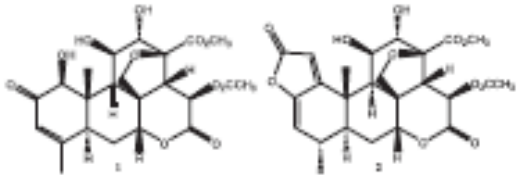

Figure 1. Quassinoids isobrucein B (**1**) and neosergeolide (**2**) present in infusions of *Picrolemma sprucei*.

Experimental

Collection of plant materials

Collection was performed in Silves, Amazonas State, Brazil, in April, 2003. Voucher specimens are on deposit at the UFAM Herbarium (Silva 5729 & 5730). Identification of *P. sprucei* Hook. f. was performed by Dr. Wayt Thomas.[18]

Chemicals and reagents

Methanol, HPLC grade, was purchased from J.T.Baker (Xalostoc, Mexico). Glacial acetic acid was of analytical grade and supplied by Merck (Darmstadt, Germany). Ultrapure water, obtained from a Milli-Q Plus System (Millipore, Bedford, MA, USA), was used in all analyses. **1** and **2** were isolated from *P. sprucei* as described previously[11,19] and used as reference standards. Their identity and purity were established based on 1D and 2D NMR, IR, HR-ESI-TOF-MS, HPLC-DAD and LC-ESI-MS analyses and comparison to literature data.[20]

Standard solutions

Stock standard solutions were prepared at 1 mg mL^{-1} in methanol and stored at -20 °C for a maximum of three months. Working solutions were prepared by appropriate dilution with methanol to have final concentrations in the ranges 0.25 to 5 μg mL^{-1} and 0.5 to 10 μg mL^{-1} for **1** and **2**, respectively. Working solutions had the internal standard (IS) phloroglucinol (1,3,5-trihydroxybenzene) at a concentration of 4.0 μg mL^{-1}.

LC and MS detection conditions

The liquid chromatography system consisted of an LC-10AD pump and a CTO-10AS column oven from Shimadzu (Kyoto, Japan). The separation of **1**, **2** and IS were performed on a reversed phase NST18 column, 250 mm × 4.6 mm ID, 5 µm particle size (Nano Separation Technologies, São Carlos, São Paulo, Br). A LiChrospher® 100 RP 18 column, 4 mm × 4 mm ID, 5 µm particle size, from Merck (Darmstadt, Germany) was used as guard column. The LC system was operated isocratically using mobile phase consisting of a mixture of methanol:water (50:50, v/v) and 2 % acetic acid which was pumped at a flow-rate of 0.8 mL. min^{-1}. Injections of samples were performed manually through a 20 µL loop with a Rheodyne model 7125 injector (Rheodyne, Cotati, USA) and the column was kept in an oven set at 27±1 °C.

Detection was achieved with a Quattro Micro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray interface (ESI) source and tandem mass separation operated in the positive ion mode at a potential of 2.5 kV. The LC flow was split so that approximately 150 µL. min^{-1} entered the mass spectrometer. The desolvation temperature was set to 250 °C and the source was set to 100 °C for LC-MS. The nitrogen desolvation and nebulizer gas flow rates were set to 250 and 20 L h^{-1}, respectively. Argon gas was used as collision gas. Cone voltage and collision energy were optimized for each analyte by performing full scan acquisitions. Optimization of MS conditions was achieved by direct infusion of standard solutions (10 µg mL^{-1}) prepared in the mobile phase and delivered by a syringe pump at a flow-rate of 10 µL. min^{-1}. The cone voltage was set at 40 V for **1** and **2** and 20 V for IS, and collision energies of 20 eV for **1** and IS and 28 eV for **2** were used. For quantitation, MS/MS was operated in the multiple reaction monitoring (MRM) mode, monitoring the transitions 481>403, 505>225 and 127>81 for **1**, **2** and IS, respectively, with a dwell time of 1.0 s. Data acquisition and quantitative analysis were performed using a MassLynx (Micromass) data acquisition system, version 4.1.

Validation of the method

The method was rigorously validated in accordance with United States Food and Drug Administration (FDA) guidelines[22] by determination of the following parameters: linearity, range, precision, accuracy, limit of quantification (LOQ) for LC-MS/MS. For linearity and range, the calibration curves were constructed by injection of seven concentrations of **1** and **2**, ranging from 0.25 to 5.0 µg mL^{-1} and 0.5 to 10 µg mL^{-1}, respectively, in triplicate.

The analyte to IS peak area ratio was plotted against the respective standard concentrations and the linearity was evaluated by linear regression analysis. The acceptance criterion for each calculated standard concentration was a maximum 2% deviation from the nominal value, except for the LOQ, which was set as 10 % deviation. The precision of the method was determined by evaluating repeatability and LOQ was established using the criterion that responses of the analytes at the LOQ should be at least 5 times that of the blank response.

Sample preparation

5.00 g of dry, ground stems of *Picrolemma spruceii* were infused in 1.00 L of boiling water. The resulting mixture was covered, allowed to stand for 15 min, then immediately filtered. The filtrate was evaporated to dryness under vacuum which resulted in a residue. The residue was dissolved in methanol in an ultrasound bath which provided a solution (10.0 mL) which was filtered. The resulting filtrate or RSI (residue of stem infusion in methanol solution) was stored at –20 °C and IS was added prior to LC-MS analysis. The concentration of quassinoids was calculated from the linear regression equations.

Results and Discussion

Development of LC-ESI-MS conditions

In order to quantify **1** and **2** in an infusion of stems of *P. spruceii* used in Brazilian folk medicine, MS parameters were optimized in order to obtain more abundant precursor ions. Thus, the balance of protonated or deprotonated and cationized or anionized **1** and **2** or other adduct ion species of **1** and **2** were analyzed. Ideally, these species should be of high intensity to allow analytes to be detected at low concentrations in complex matrices. The intensity of ion species was therefore the main criteria for optimization. Initially, **1** and **2** were directly infused into the MS detector using ESI ionization. Both positive and negative ionization modes of the ESI source were tested by infusion of standard solutions of each compound at 10 µg mL^{-1}. The positive ion detection mode offered higher sensitivity than the negative mode using an acidic mobile phase. (+)-ESI-MS full scan spectra displayed protonated ions ($[M+H]^+$) with good intensities at *m/z* 481 and 505 for **1** and **2**, respectively.

During the optimization of the method, different LC conditions and solvent systems were evaluated. Addition of acidic modifiers such as acetic acid to the mobile phase was found to improve peak resolution. Furthermore, the positive ion detection mode [(+)-ESI] offered higher sensitivity than

negative ion mode [(-)-ESI] when acidic mobile phase was used. Also, addition of acetic acid or formic acid to the mobile phase led to significant enhancement of the $[M+H]^+$ ions and significant reduction of the cationized molecules. Table 1 presents the parent and fragment ions used for identification and quantification of compounds **1** and **2**.

Table 1. Protonated molecule and fragment ions obtained in ESI-MS/MS spectra. The most stable and intensive ions (*) were used for identification and quantification of isobrucein B (1) and neosergeolide (2) by LC-ESI-MS/MS

	Quassinoid	
	1	**2**
Protonated molecule $[M+H]^+$	**481**	**505**
Fragment ions	463	487
	445	463
	439	445
	421	427
	403*	417
	391	413
	375	398
	357	379
	356	367
	311	348
	301	339
	299	323
	284	321
	279	319
	214	227
		225*

Stem infusion in solution (RSI) was directly injected into the LC-ESI-MS/MS system. Figure 2 presents the representative MRM-chromatogram. Under the conditions described in the method, IS, **1** and **2** eluted at retention times of 3.5, 4.6 and 8.6 min, respectively, and elution of all components of the sample was complete within 10 min. Chromatographic profiles displayed good separation of the analyte components in a short period of time.

Method validation

The validation parameters were evaluated through estimation of linearity, precision (intra and inter day) and accuracy of the method. The linearity of an analytical method is its ability, within a definite range, to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample. The calibration curve for each analyte was based on 7 different concentrations by plotting

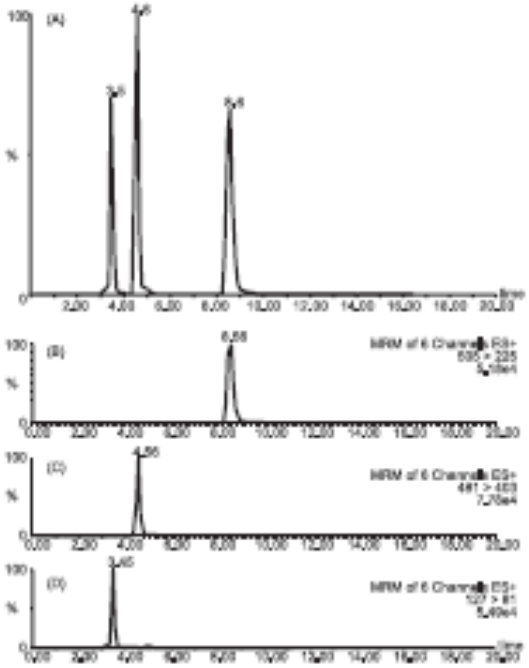

Figure 2. Representative chromatogram. (A) Total ion count. (B) MRM of neosergeolide (2), (C) MRM of isobrucein B (1) and (D) MRM of the internal standard phloroglucinol (1,3,5-trihydroxybenzene). Chromatographic conditions: reversed phase NST18 column, 250 mm x 4.6 mm ID, 5 µm. Eluents were methanol:water (50:50, v/v) and 2% acetic acid. Flow rate was 0.8 mL min^{-1}. Injection volume was 20 µL. Column oven temperature was set at 27±1 °C.

the result of the linear regression analysis of the peak area ratio versus concentration.

Linear regression analysis provided the equations presented in Tables 2 and 3. For both **1** and **2**, the correlation coefficients (r^2) were evidence for a linear relationship between concentration and peak area ratio in the concentration range studied. The values of LOQ are also presented in tables 2 and 3 and were considered satisfactory for both analytes.

Application of the method

In order to check the applicability of the developed method, a stem infusion was evaluated. This particular preparation was chosen based on the wide availability of stems in marketplaces in the western Brazilian Amazon and the use of this preparation as an antimalarial in traditional medicine in this region. Concentrations of **1** and **2** in samples of stem infusion were calculated based on the integration of the $[M+H]^+$ peak area ratio of each of these analytes in the MRM chromatogram. By this process it is possible to select the compound of interest and quantification of the

Table 2. Confidence limits for quantification of isobucuzin B with the method developed

Parameters	Isobucuzin B		
Accuracy and Precision			
Concentration added (μg mL^{-1})	0.25	1.0	3.0
CV, n=6 (%)	1.1	1.2	1.6
Within-day precision, (CV, %)	1.5	1.7	1.8
Within-day accuracy[a] (%)	1.5	1.8	1.2
Linearity			
Range (μg mL^{-1})	0.25 - 5.0		
Linear regression	y = 0.8964x + 0.1958		
Correlation coefficient	0.9909		
Quantitation Limit			
Concentration (μg mL^{-1})	0.25		
Within-day precision, n=5 (CV, %)	5.2		
Within-day accuracy[a], n=5 (%)	4.6		

[a] Expressed as relative standard deviation, RSD

Table 3. Confidence limits for quantification of neosergeolide with the method developed

Parameters	Neosergeolide		
Accuracy and Precision			
Concentration added (μg mL^{-1})	1.5	3.0	5.0
CV, n=6 (%)	1.4	1.6	0.8
Within-day precision, (CV, %)	1.3	1.8	1.5
Within-day accuracy[a] (%)	0.9	1.3	1.4
Linearity			
Range (μg mL^{-1})	0.5 - 10.0		
Linear regression	y = 0.5972x + 0.5928		
Correlation coefficient	0.9948		
Quantitation Limit			
Concentration (μg mL^{-1})	0.50		
Within-day precision, n=5 (CV, %)	7.8		
Within-day accuracy[a], n=5 (%)	6.3		

[a] Expressed as relative standard deviation, RSD

individual compounds (considering different molar masses and fragment ions) can be performed without the need for complete chromatographic resolution, but in this case good chromatographic resolution was observed. By this method, **1** and **2** were determined to be present at concentrations of 0.601 and 7.74 μg mL^{-1}, respectively, in the sample solution of stem infusion (RSI) analyzed, which corresponds to concentrations of 60.1 and 774 μg L^{-1}, respectively, of these quassinoids in the infusion.

Conclusion

The LC-(+)-ESI-MS/MS method described herein proved suitable for the analysis of **1** and **2** in stem extracts and can be easily applied in routine analyses. Furthermore, the method may be used to certify the quality of popular medicinal uses of the extract and for ecological studies.

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CAPÍTULO 6: Envolvimento de um caminho intrínseco mitocondrial na apoptose induzida pela neosergeolida de células leucêmicas HL-60: O papel do poro de transição e permeabilidade mitocondrial e danos ao DNA.

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RESEARCH ARTICLE

Involvement of intrinsic mitochondrial pathway in neosergeolide-induced apoptosis of human HL-60 leukemia cells: The role of mitochondrial permeability transition pore and DNA damage

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Abstract

Context: Quassinoids are biologically active secondary metabolites found exclusively in the Simaroubaceae family of plants. These compounds generally present important biological properties, including cytotoxic and antitumor properties.

Objective: In the present study, the cytotoxic effects of neosergeolide, a quassinoid isolated from *Picrolemma sprucei* Hook. f., were evaluated in human promyelocytic leukemia cells (HL-60).

Materials and methods: Cytotoxicity and antiproliferative effects were evaluated by the MTT assay, May-Grünwald-Giemsa's staining, BrdU incorporation test, and flow cytometry procedures. The comet assay and micronuclei analysis were applied to determine the genotoxic and mutagenic potential of neosergeolide.

Results: After 24 h exposure, neosergeolide strongly inhibited cancer cell proliferation (IC_{50} 0.1 μM), and its activity seemed to be selective to tumor cells because it had no antiproliferative effect on human peripheral blood mononuclear cells (PBMC) at tested concentrations. Apoptosis was induced at submicromolar concentrations (0.05, 0.1, and 0.2 μM) as evidenced by morphological changes, mitochondrial depolarization, phosphatidylserine externalization, caspases activation, and internucleosomal DNA fragmentation. Additionally, neosergeolide effects were prevented by cyclosporine A (CsA), an inhibitor of the mitochondrial permeability transition (MPT) pore, which reinforced the participation of intrinsic pathways in the apoptotic process induced by this natural quassinoid. Direct DNA damage was further confirmed by comet assay and cytokinesis-block micronucleus test.

Discussion and conclusion: The present study provided experimental evidence to support the underlying mechanism of action involved in the neosergeolide-mediated apoptosis. In addition, no antiproliferative effect or DNA damage effect of neosergeolide was evident in PBMC, highlighting its therapeutic potential.

Keywords: Neosergeolide, quassinoid, cytotoxicity, apoptosis

Introduction

Quassinoids are degraded triterpenes frequently highly oxygenated (Polonsky et al., 1980; Iiwajinda et al., 2002), found exclusively in plants belonging to the Simaroubaceae family. These compounds generally presented important biological properties (Okano et al., 1990; Guo et al., 2005a), including antimalarial (Guo et al., 2005b; Bertani et al., 2006; Silva et al., 2009),

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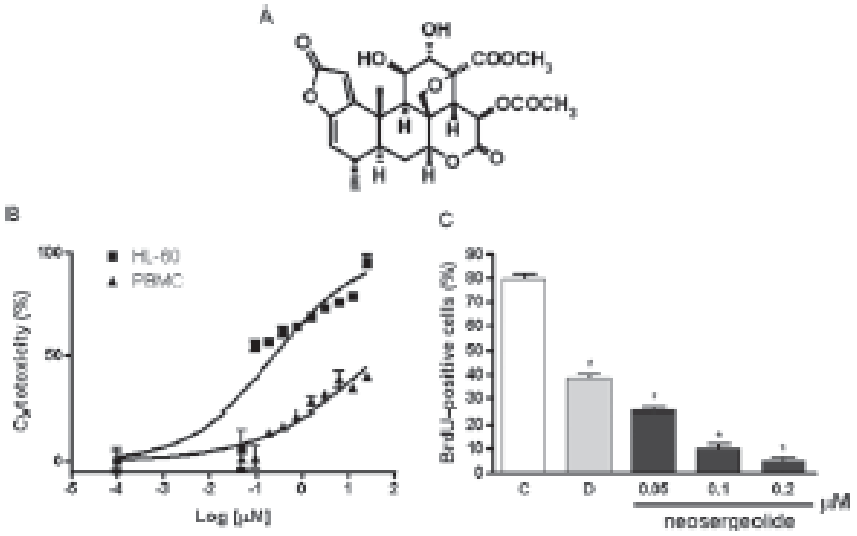

Figure 1. Chemical structure of neosergeolide (A), concentration-response curve of neosergeolide cytotoxicity (%) after 24 h exposure of HL-60 and PBMC (B), and its effects on 5-bromo-2-deoxyuridine (BrdU) incorporation (C) by HL-60 cells. *$p < 0.001$ compared to control by ANOVA followed by Student Newman–Keuls test. Data are presented as means ± S.E.M. for three independent experiments in triplicate.

anti-HIV (Okano et al., 1996), and cytotoxic and antitumor activities (Tischler et al., 1992; Itokawa et al., 1993; Mata-Greenwood et al., 2001; Murakami et al., 2004; von Bueren et al., 2007; Silva et al., 2009). Since the use of bruceantin (Kupchan et al., 1973) in phase II breast cancer and melanoma clinical trials and its subsequent withdrawal due to the concerns about toxicity (Wiseman et al., 1982; Arseneau et al., 1983), studies on the antitumor properties of quassinoids have increased steadily (Okano et al., 1985; Mata-Greenwood et al., 2001; Guo et al., 2005a).

Recently, it has been reported that neosergeolide (Figure 1A), a quassinoid isolated from *Picrolemma sprucei* Hook. f. (Simaroubaceae), has significant *in vitro* antimalarial, larvicide, and cytotoxic properties (Silva et al., 2009). Also, neosergeolide nonselectively inhibited cancer cell lines proliferation (SF295 (glioblastoma), MDA-MB435 (melanoma), HCT8 (colon), and HL-60 (leukemia)) as evidenced by quite similar IC_{50} values. In the present study, the underlying molecular mechanisms of neosergeolide's antiproliferative activity were evaluated using human promyelocytic leukemia cells (HL-60). The HL-60 cells are extensively used in the examination of the effects of test drugs on cell proliferation, cell cycle, cell differentiation, and apoptosis events (Collins, 1987; Militão et al., 2006). For comparison, the effect of neosergeolide on the growth of normal cells was evaluated using peripheral blood mononuclear cells (PBMC).

Materials and methods

Drugs and reagents

The procedure used for the isolation of neosergeolide from the roots and stems of *P. sprucei* was described in a previous publication (Andrade-Neto et al., 2007). Neosergeolide used in this study had purity greater than 98% based on HPLC (DAD/ESI-MS) and NMR analyses. Fetal calf serum and phytohemagglutinin were purchased from Cutilab (Campinas, SP, Brazil). RPMI 1640 medium, trypsin-EDTA, penicillin, and streptomycin were purchased from GIBCO® (Invitrogen, Carlsbad, CA, USA). Cytochalasin-B (Cyt-B), cyclosporine A (CsA), sulfanilamide, rhodamine 123 (Rho-123), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Doxorubicin (Doxolem®) was purchased from Zodiac Produtos Farmacêuticos S. A. (São Paulo, SP, Brazil). All other chemicals and reagents were of analytical grade.

Cell cultures

The human promyelocytic leukemia cell line (HL-60) was donated by the National Cancer Institute (Bethesda, MD, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C with 5% CO_2. Heparinized blood from healthy, non-smoker donors who had not taken any drug at least 15 days prior to sampling was collected and PBMC were isolated using density-gradient centrifugation over Histopaque-1077. The PBMC were washed and re-suspended in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C under a 5% CO_2 atmosphere. Phytohemagglutinin (2%) was added at the start of cultures. After 24 h, cells were treated with the test compounds.

Neosergeolide toxicity to HL-60 cells and PBMC

The cytotoxicity of neosergeolide to HL-60 cells and PBMC was evaluated by the MTT assay (Mosmann,

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1983). Briefly, cells were plated in 96-well plates (3×10^5 cells/mL for HL-60 and 4×10^5 cells/mL for PBMC). Neosergeolide was dissolved in 1% DMSO at concentrations of 0.006–4 μM and the resulting solutions were added to wells. After 24 h, the supernatant was replaced by fresh medium containing MTT (0.5 mg/mL). After 3 h, the MTT formazan product was dissolved in DMSO and absorbance was measured at 595 nm (Beckman Coulter® DTX-880 spectrometer). Doxorubicin (0.044–46 μM) was used as positive control.

Antiproliferative effect (inhibition of BrdU incorporation)

The HL-60 cells were plated in 24-well tissue culture plates (3×10^5 cells/mL) and treated with neosergeolide at different concentrations. After 21 h of exposure to sample, 20 μL of BrdU (10 mM) was added to each well and incubated for 3 h at 37°C. To determine the amount of BrdU incorporated into DNA (Pera et al., 1977), cells were harvested, transferred to cytospin slides and allowed to dry for 2 h at room temperature. Cells that had incorporated BrdU were labeled by direct peroxidase immunocytochemistry using the chromogen diaminobenzidine (DAB). Slides were counterstained with hematoxylin, mounted, and coverslipped. Determination of BrdU positivity was performed by light microscopy (Olympus, Tokyo, Japan). A total of 200 cells were counted per sample to determine the percentage of BrdU-positive cells. Doxorubicin (0.6 μM) was used as positive control.

Analysis of morphological changes

Untreated or neosergeolide-treated HL-60 cells were examined for morphological changes by light microscopy (Metrimpex Hungary/PZO-Labimex Modelo Studar Lab®). To evaluate morphology, cells were harvested, transferred to cytospin slides, fixed with ethanol for 1 min and stained with May-Grünwald-Giemsa. Doxorubicin (0.6 μM) was used as positive control.

Flow cytometric experiments

Cell membrane integrity

The HL-60 cell membrane integrity was evaluated by the exclusion of propidium iodide (PI) at 50 μg/mL. Aliquots were removed from cultures after 3, 6, 12, and 24 h of incubation. Cell fluorescence was then determined by flow cytometry in a Guava EasyCyte Mini (Guava Technologies, Inc., Hayward, CA, USA) using Guava Express Plus software. A total of 5,000 events were evaluated per experiment.

Internucleosomal DNA fragmentation

Aliquots were removed from HL-60 cell cultures after 3, 6, 12, and 24 h of incubation with neosergeolide. Then, the aliquots were incubated at 37°C for 30 min in the dark in a lysis solution containing 0.1% citrate, 0.1% Triton X-100, and 50 μg/mL PI. Cell fluorescence was then determined by flow cytometry in a Guava EasyCyte Mini (Guava Technologies, Inc., Hayward, CA, USA) using Guava Express Plus software. The percentage of degraded DNA was determined by the number of cells displaying sub-diploid (sub-G_0/G_1) DNA divided by the total number of cells examined. A total of 5,000 events were evaluated per experiment.

Mitochondrial transmembrane potential ($\Delta\psi_m$)

Mitochondrial depolarization was evaluated after 3, 6, 12, and 24 h of incubation with neosergeolide using the method of incorporation of Rho-123. Rho-123 is a cell-permeable, cationic, fluorescent dye that is readily sequestered by active mitochondria without inducing cytotoxic effects. Briefly, treated and untreated HL-60 cells were centrifuged at 2000 rpm for 5 min and the pellet was re-suspended in 200 μL of a 1 μg/mL solution of Rho-123 for 15 min in the dark. After incubation, cells were centrifuged at 2000 rpm for 5 min. The resulting pellet was re-suspended in 200 μL of phosphate-buffered saline (PBS) and incubated for 30 min in the dark. Fluorescence was measured and percentage of mitochondrial depolarization was determined (Cury-Boaventura et al., 2004).

Annexin V/PI and caspases (9, 3, and 7) detection

The Annexin V (AnnV) cytometry assay was used to detect cell population in viable, early and late apoptosis stage. After short exposure time (3 h) or pulse treatment (3 h of neosergeolide exposure following 21 h reincubation period without drug), HL-60 cells were stained with fluorescein isothiocyanate (FITC) conjugated AnnV (Guava Nexin kit, Guava Technologies, Inc., Hayward, CA, USA) and PI (necrotic-cell indicator), and then they were subjected to flow cytometry (Guava EasyCyte Mini). Cells undergoing early and late apoptosis were detected by the emission of the fluorescence from only FITC and, both FITC and PI, respectively. Also, the percentage of cells with active caspases (9, 3, and 7) were estimated by flow cytometry (Guava EasyCyte Mini) using a Guava Caspases 9 and 3/7 FAM Kit (Guava Technologies, Inc., Hayward, CA, USA).

Analysis of mitochondrial permeability transition on neosergeolide-induced cell death

To corroborate the central role of mitochondria in the apoptotic process induced by neosergeolide, cells were pretreated or not for 30 min with CsA at 5 μM [a blocker of mitochondrial permeability transition (MPT)] prior to the neosergeolide exposure and cotreated for 3 h. After exposure time, mitochondrial depolarization, internucleosomal DNA fragmentation, active caspases (9, 3, and 7) detection, and morphological analysis of cell death (AO/EB staining) were performed as described above.

Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) were estimated after treatment with neosergeolide using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) as fluorescence probe. The H_2DCFDA diffuses through

the cell membrane readily and is hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescein (DCFH), which is then rapidly oxidized to highly fluorescent DCFH (2′,7′-dichlorofluorescein) by a broad range of intracellular oxidative stresses other than H_2O_2 (Crow, 1997; Hempel et al., 1999). Therefore, increased mean fluorescence intensity of DCF represents a probe of oxidation by a broad range of oxidative events and not only reaction with H_2O_2. At different exposure times (3 and 24 h), the culture medium was replaced by fresh serum-free medium containing 20 µM H_2DCFD. DCF fluorescence intensity was detected by flow cytometry using a Guava EasyCyte Mini (Guava Technologies, Inc., Hayward, CA, USA) and Guava Express Plus software. The DCF fluorescence intensity is proportional to the amount of intracellularly formed ROS (LeBel et al., 1992).

DNA damage analysis

Micronuclei assay

After pulse treatment (3 h), HL-60 cells were incubated in complete medium for 48 h and Cyt-B (3 µg/mL) was treated at 24 h. Cells were harvested and re-suspended in a hypotonic solution (0.075 M KCl) for 10 min. Afterward, HL-60 cells were harvested again and Carnoy's fixative was added gently. Then, cells were dropped onto clean slides and air-dried. These slides were stained with 8% Giemsa solution (pH 6.8) and then observed under a light microscope. Micronuclei (MN) were reported per 1,000 binucleated cells (BNC) with well-preserved cytoplasm (Eckhardt et al., 1994). To verify the genotoxic potential of neosergeolide against PBMC, the same protocol used for leukemia cells was applied.

Alkaline comet assay

The comet assay was conducted under alkaline conditions as described by Singh et al. (1988) with modifications (Klaude et al., 1996) and following the recommendations of the International Workshop on Genotoxicity Test Procedures (Tice et al., 2000). After pulse treatment (3 h), HL-60 cells were collected and processed for the assay as follows. Briefly, 15 µL of the cell suspension were mixed with 90 µL of 0.75% low melting point agarose in PBS at 37°C; 100 µL of the cell suspension were spread on a glass slide previously coated with a layer of 1.5% normal melting point agarose in PBS, covered with a glass coverslip and placed at 4°C for 15 min. The coverslip was gently removed and the slide was submerged into ice-cold lysing solution (2.5 M NaCl, 10 mM Tris, 0.1 mM EDTA, 1% sodium sarcosinate, 1% Triton X-100, and 10% DMSO, pH 10) at 4°C for at least 1 h. After lysis, the slides were placed in a horizontal gel electrophoresis chamber with freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH >13.0). The slides were kept in this solution for 20 min at 4°C to allow unwinding of the DNA and expression of alkali-labile sites. Then, the samples were subjected to electrophoresis in the same solution at 300 mA, 0.81 V/cm for 20 min at 4°C. After electrophoresis, the slides were rinsed gently three times (5 min each time) with 0.4 M Tris-HCl (pH 7.5). Each slide was stained with 50 µL of ethidium bromide (20 µg/mL) and covered with a coverslip. The analysis of the cells was performed by a visual scoring system (Miyamae et al., 1998). Briefly, fluorescently stained nucleoids were scored visually using an epifluorescence microscope (Olympus, Tokyo, Japan) with an excitation filter of 510–560 nm and a barrier filter of 590 nm at 400× magnification.

A total of 300 randomly selected cells (100 cells from each of the three replicate slides) were analyzed for each concentration of test substance. Cells were scored visually according to tail length into five classes: (1) class 0: undamaged cells having no tail; (2) class 1: cells having a tail shorter than the diameter of the head (nucleus); (3) class 2: cells having a tail length 1–2 times the diameter of the head; (4) class 3: cells having a tail longer than 2 times the diameter of the head; (5) class 4: comets having no heads. A value (damage index, DI) was assigned to each comet according to its class, using the formula:

$$DI = (0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4),$$

where n = number of cells in each class analyzed. Damage index thus ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) (Collins et al., 1995; Silva et al., 2000). Doxorubicin (0.6 µM) was used as positive control. To assess DNA damage in PBMC caused by neosergeolide, the same protocol used for leukemia cells, as described above, was applied.

Statistical analysis

Data are presented as means ± S.E.M. IC_{50} values and 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA, USA). For all experiments, data were analyzed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test.

Results

Neosergeolide reduces both proliferation and viability of HL-60 cells

The MTT-based assay showed that neosergeolide exhibited elevated cytotoxicity to HL-60 cells after 24 h. Data from two independent experiments carried out in triplicate provided an IC_{50} value of 0.1 µM (IC_{95} 0.06–0.15 µM). Neosergeolide was not cytotoxic to PBMC (IC_{50} > 4.0 µM) at tested concentrations. Doxorubicin was used as positive control and displayed potent cytotoxicity against HL-60 cells (IC_{50} 0.04 µM, CI 95% 0.02–0.04 µM) and PBMC (IC_{50} 0.33 µM, CI95% 0.20–0.44 µM). Subsequent experiments were conducted at concentrations corresponding to $1/2 \times IC_{50}$, IC_{50}, and $2 \times IC_{50}$ (0.05, 0.1, and 0.2 µM, respectively).

To further study the inhibition of proliferation, the incorporation of the nucleotide BrdU into DNA was evaluated by direct peroxidase immunocytochemistry in treated and

untreated HL-60 cells. After 24 h, neosergeolide at the concentrations of 0.05, 0.1, and 0.2 μM inhibited BrdU incorporation by 48.00±2.30, 89.67±1.45, and 94.67±1.45%, respectively, in HL-60 cells (Figure 1B; $p<0.001$).

Neosergeolide alters cell morphology, inducing both apoptosis and necrosis in HL-60 cells

To determine whether growth inhibition was related to the induction of apoptosis and necrosis, morphological analysis of neosergeolide-treated HL-60 cells was carried out using May-Grünwald-Giemsa staining.

Analysis of May-Grünwald-Giemsa stained neosergeolide-treated and untreated HL-60 cells revealed several drug-induced morphological changes. Control cells exhibited a typical non-adherent and vacuolization round morphology after 24 h in culture (Figure 2A). Neosergeolide at all concentrations induced DNA fragmentation, reduction in cell volume, and destabilization of the plasma membrane. Progression toward cell death in a dose-dependent manner was observed (Figure 2C–2E). Also, pyknotic nuclei were observed at all concentrations. The positive control substance doxorubicin (0.6 μM) also induced reduction in cell volume, besides nuclear fragmentation and destabilization of the plasma membrane (Figure 2B).

Effects of neosergeolide on cell membrane integrity, cell proliferation, internucleosomal DNA fragmentation, and $\Delta\psi_m$ by flow cytometry

Proliferation and cell membrane integrity were determined by the exclusion of PI (Figure 3A and 3B). In HL-60 cell cultures treated with 0.05 and 0.1 μM neosergeolide, loss of membrane integrity was observed only after 24 h ($p<0.05$). At 0.2 μM, neosergeolide induced early loss of membrane integrity, which began after 6 h of exposure. Neosergeolide reduced the number of viable cells in a time- and concentration-dependent manner. Cultures exposed to neosergeolide at low concentration (0.05 μM) showed a decrease in cell viability only after 12 and 24 h of treatment ($p<0.05$). However, at the highest concentration (0.2 μM), neosergeolide reduced cell viability at all exposure times evaluated. Doxorubicin reduced the number of viable cells (Figure 3B) without membrane damage (Figure 3A).

At all concentrations tested, neosergeolide caused cell shrinkage and nuclear condensation as evidenced by decrease in forward light scattering and transient increase in side scattering, respectively. Both of these morphological modifications are compatible with the presence of apoptotic cells. All subdiploid-sized DNA (sub-G_0/G_1) was considered to be due to internucleosomal DNA

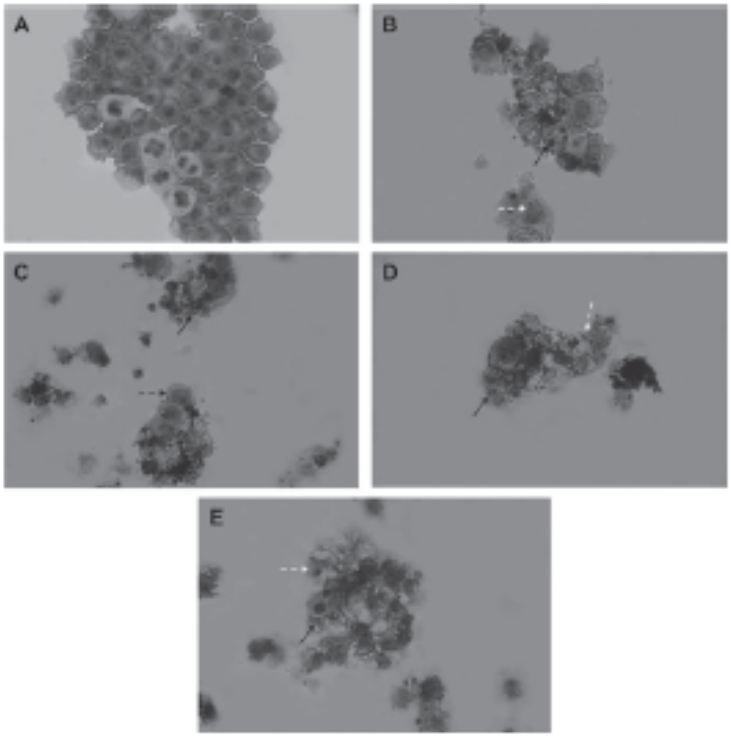

Figure 2. Morphological features induced by neosergeolide. Microscopic analysis of untreated (A) and neosergeolide-treated HL-60 cells (C–0.05 μM, D–0.1 μM and E–0.2 μM). Doxorubicin (0.6 μM) was used as positive control (B). Cells were incubated for 24 h and stained by May-Grünwald-Giemsa. Black arrows: nuclei pyknotic and nuclear fragmentation, white dashed arrows: debris, and black dashed arrow: membrane damage. Magnification: 400×.

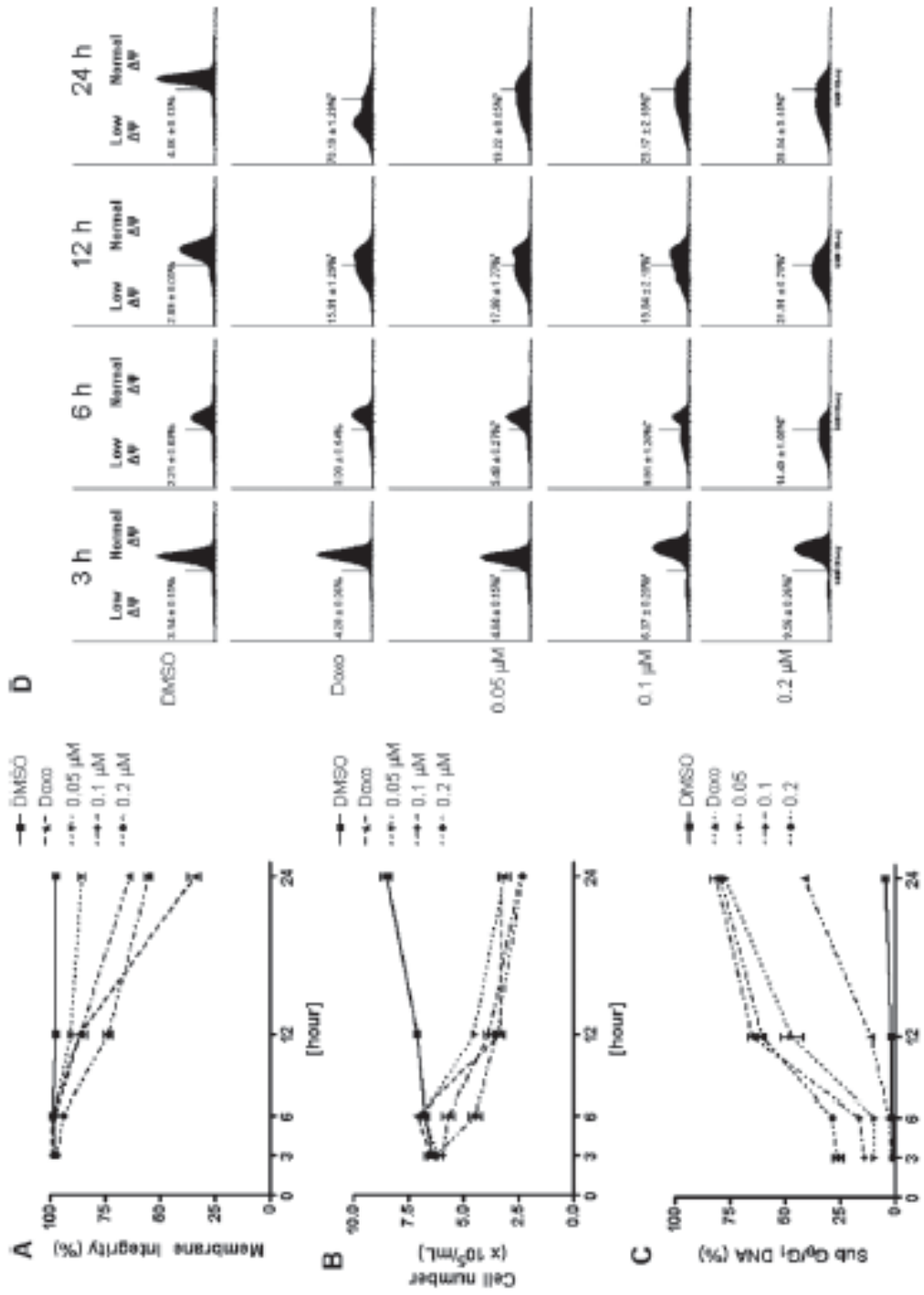

Figure 3. Effect of neosergeolide on HL-60 cell membrane integrity (A), proliferation (B), internucleosomal DNA fragmentation (C), and mitochondrial transmembrane potential (D), determined by flow cytometric analysis after 3, 6, 12, and 24 h of incubation. The vehicle (0.1% DMSO) used for diluting the test substance was used as negative control. Doxorubicin at 0.6 μM was used as positive control. A total of 5,000 events were analyzed in each experiment. *$p<0.001$; **$p<0.05$ compared to control by ANOVA followed by Newman–Keuls test. Data are represented as mean values ± S.E.M. from three independent experiments, in triplicate.

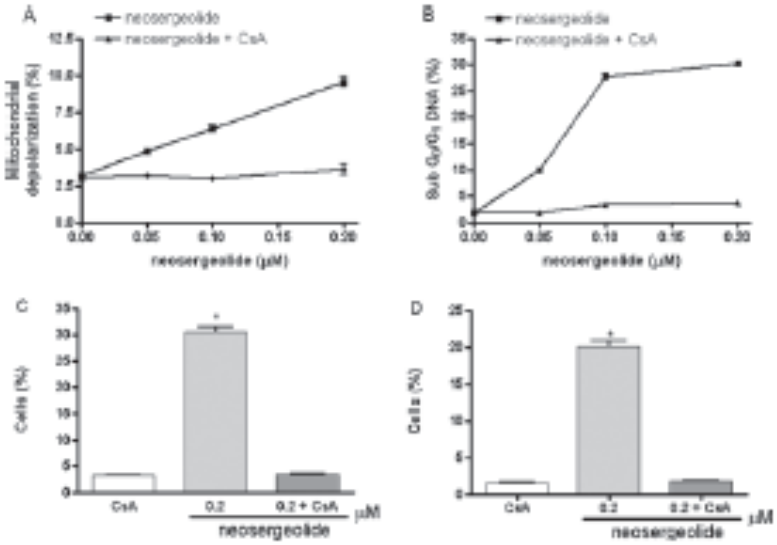

Figure 4. Effect of neosergeolide after 3 h exposure in the presence or absence of CsA (5 μM) on HL-60 mitochondrial transmembrane potential (A), internucleosomal DNA fragmentation (B), percentage of cells with active caspase-9 (C), and caspases-3 and -7 (D) determined by flow cytometric analysis.

fragmentation. Increased neosergeolide-induced apoptotic sub-G_0/G_1 peaks represent apoptotic cells having fractional DNA content and were observed at all concentrations 3, 6, 12, and 24 h after treatment (Figure 3C; $p < 0.001$). Doxorubicin also induced apoptotic effects.

Neosergeolide-induced mitochondrial depolarization in HL-60 cells was evidenced by Rho-123 incorporation after 3, 6, 12, and 24 h of incubation (Figure 3D). These data suggest that neosergeolide induces apoptosis in HL-60 cells by triggering an intrinsic mitochondrial pathway.

Protection by CsA against neosergeolide-induced cell killing in HL-60

As MPT and caspases play crucial roles in the process of apoptosis, the effects of CsA, a specific inhibitor of MPT, on neosergeolide-induced apoptosis was investigated. After 3 h exposure, neosergeolide reduces the mitochondrial depolarization (Figure 4A) and causes an increase in the DNA fragmentation (sub-G_0/G_1 peaks) (Figure 4B) as well in the number of cells with activate caspase-9 (Figure 4C) and caspases-3 and -7 (Figure 4D). To implicate a role of the MPT in neosergeolide-induced cell killing, HL-60 cells were pretreated with 5 μM CsA before exposure to neosergeolide. The CsA completely blocked neosergeolide-induced apoptosis (Figure 4A–4D).

Neosergeolide induces phosphatidylserine externalization and activation of caspases-3 and -7 after 3 h (pulse treatment)

To verify if neosergeolide-induced cytotoxicity was a reversible process, the cells were treated for 3 h; after short-exposure time, the drug was removed, and then the cultures were reincubated for 21 h. After 3 h pulse treatment, neosergeolide caused a significant increase in the number of early ($AnnV^+/PI^-$) and late ($AnnV^+/PI^+$) apoptotic HL-60 cells when compared to the control group (Figure 5). For AnnV/PI experiments, we did not observe a significant concentration–response relationship in the number of necrotic cells ($AnnV^-/PI^+$): 0.12 ± 0.01, 0.04 ± 0.01, 0.46 ± 0.02, 3.40 ± 0.98, and 1.90 ± 0.22%, for negative control (vehicle) cultures, doxorubicin-treated cultures, and 0.05, 0.1, and 0.2 μM neosergeolide-treated cultures, respectively. Corroborating with our AnnV/PI data, neosergeolide increases the percentage of apoptotic (early and late stages) HL-60 cells with caspases-3 and -7 activated, and a slight increase of necrotic cells population was observed: 0.70 ± 0.31 (vehicle cultures), 2.67 ± 0.25 (doxorubicin cultures), and 0.67 ± 0.21, 2.30 ± 0.56, and 3.46 ± 1.15% for 0.05, 0.1, and 0.2 μM neosergeolide-treated cultures, respectively (Figure 6).

Neosergeolide induces changes in the HL-60 cell cycle and DNA damage after 3 h (pulse treatment)

Table 1 shows the effect of neosergeolide on the HL-60 cell cycle. In general, neosergeolide at all concentrations lowered the number of cells at G_1, S, and G_2/M phases, which suggests that neosergeolide interfered in a non-specific manner in the HL-60 cell cycle. As expected, neosergeolide treatment also induces a reduction in cell proliferation, mitochondrial depolarization, and internucleosomal DNA fragmentation (sub-G_0/G_1 peaks). Also, no intracellular ROS production was detected 3–24 h after treatment with neosergeolide (data not shown).

In neosergeolide-treated HL-60 cells, the DNA damage indexes were higher than in the control cells (Figure 7A). On the other hand, no increase on DNA migration was

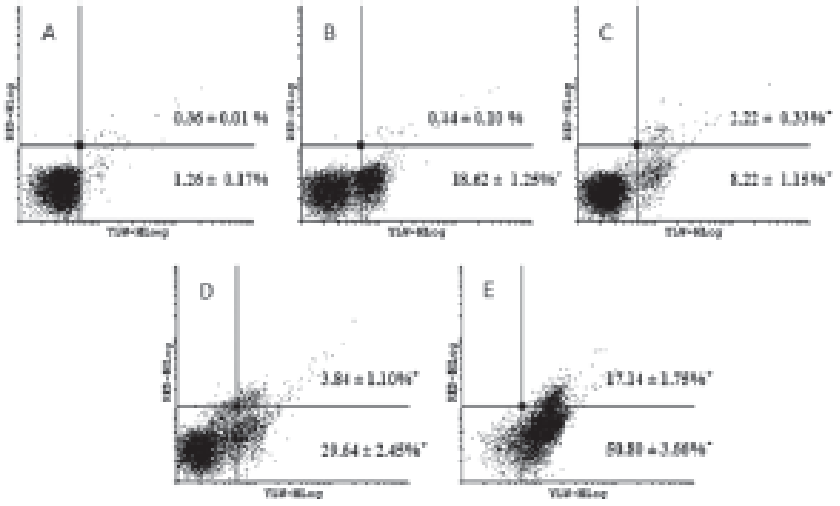

Figure 5. Effect on PS externalization after HL-60 cells treated with 0.05 (C), 0.1 (D) and 0.2 μM (E) neosergeolide. The PS externalization was determined by flow cytometry using AnnV-FITC (YLW-HLog) and PI (RED-HLog) after pulse treatment (3 h of neosergeolide exposure following 21 h reincubation period without drug). Viable cells are plotted at lower left quadrant, cells in early and late apoptosis with PS externalized are plotted at lower right and upper right quadrants, respectively, and necrotic cells are plotted at upper left quadrant. Negative control (A) was treated with the vehicle (0.1% DMSO) used for diluting the test substance. Doxorubicin (B) at 0.6 μM was used as positive control. A total of 5,000 events were analyzed in each experiment. *$p < 0.05$ compared to control by ANOVA followed by Newman–Keuls test. Data are presented as mean values ± S.E.M. from two independent experiments in triplicate.

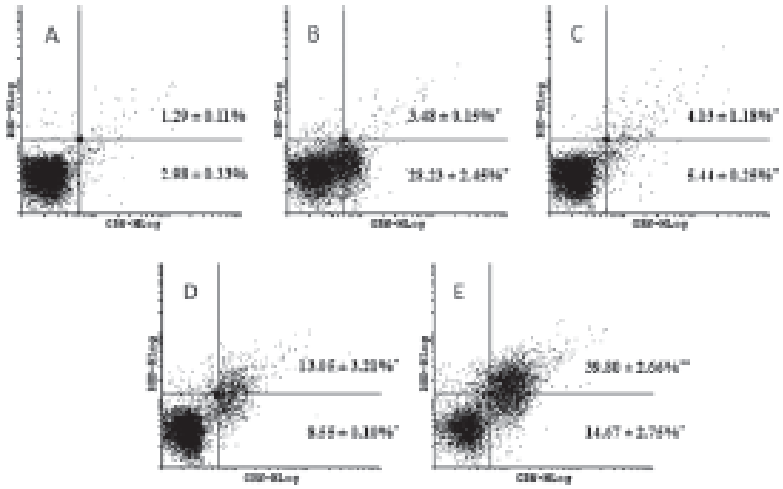

Figure 6. Effect on caspases-3 and -7 activation after HL-60 cells treated with 0.05 (C), 0.1 (D) and 0.2 μM (E) neosergeolide. Caspases activity was determined by flow cytometry using PI (RED-HLog) and fluorescent-labeled inhibitor of caspases, FLICATM (GRN-HLog) after pulse treatment (3 h of neosergeolide exposure following 21 h reincubation period without drug). Viable cells are plotted at lower left quadrant, cells in early and late apoptosis with active caspases-3 and -7 are plotted at lower right and upper right quadrants, respectively, and necrotic cells are plotted at upper left quadrant. Negative control (A) was treated with the vehicle (0.1% DMSO) used for diluting the test substance. Doxorubicin (B) at 0.6 μM was used as positive control. A total of 5,000 events were analyzed in each experiment. *$p < 0.001$; **$p < 0.05$ compared to control by ANOVA followed by Newman–Keuls test. Data are presented as mean values ± S.E.M. from two independent experiments in triplicate.

observed in neosergeolide-treated PBMC (Figure 7B). The MN frequencies were significantly increased by neosergeolide treatment at all concentrations tested ($p < 0.05$) and neosergeolide decreased cytokinesis-block proliferation of leukemia cells (Table 2; $p < 0.05$). However, the DNA damage concentrations for HL-60 cells were not genotoxic for PBMC and did not decrease the proliferation ratio of PBMC (Table 2).

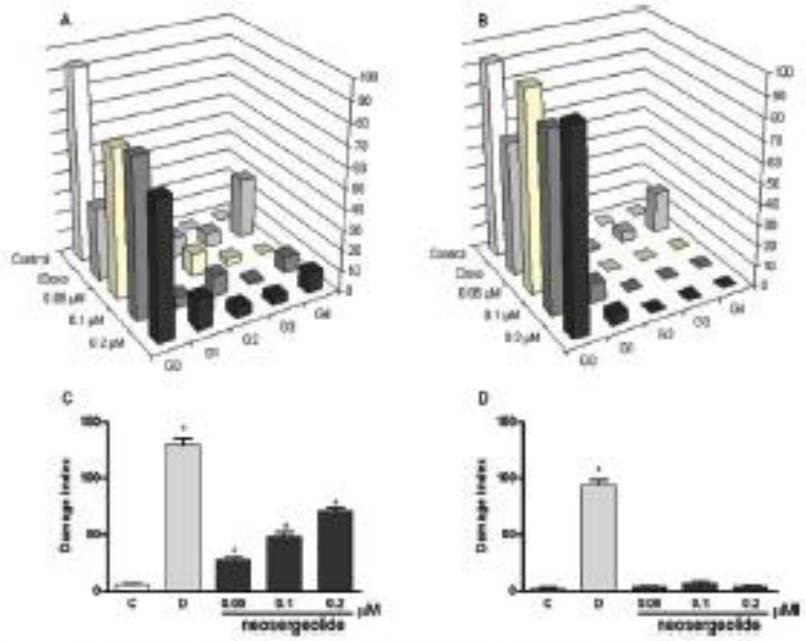

Figure 7. Effects of neosergeolide after 3 h pulse treatment on the distribution of damaged cells in alkaline comet assay into damage grades (G; grades 0–4) on HL-60 (A) and PBMC (B), its effects on HL-60 (C), and PBMC (D) DNA damage index. Bars represent the mean ± S.E.M. of three independent experiments. Negative control (C): cells were treated with the vehicle used for diluting the tested substance. Doxorubicin (0.6 µM) was used as positive control (D). *$p < 0.001$ compared to control by ANOVA followed by Newman-Keuls test.

Table 1. Effect of neosergeolide on cell cycle distribution in HL60 cells by flow cytometric using PI after 3 h pulse treatment.

Compound	Treatment (µM)	DNA content (%) Sub-G_0/G_1	G_0	S	G_2/M	Cell proliferation ($\times 10^5$/mL)	Mitochondrial depolarization (%)
C[a]	-	5.65 ± 0.29	54.24 ± 1.18	23.85 ± 0.65	14.01 ± 3.10	7.68 ± 1.29	5.59 ± 0.09
D[b]	0.6	30.60 ± 2.80**	42.40 ± 1.70**	12.36 ± 1.73*	2.06 ± 0.22**	5.42 ± 0.16*	9.76 ± 0.51**
Neosergeolide	0.05	20.74 ± 2.52**	44.03 ± 1.22**	27.21 ± 0.32*	4.63 ± 1.33**	6.60 ± 0.23	13.08 ± 0.45*
	0.1	24.92 ± 2.92**	43.26 ± 2.17**	13,21 ± 1.18*	2.80 ± 0.66**	5.13 ± 0.43*	19.21 ± 0.35*
	0.2	43.99 ± 2.99*	33.09 ± 2.02*	6.65 ± 0.70*	0.93 ± 0.18*	4.38 ± 0.59*	14.74 ± 2.34*

[a]Negative control (0.1% DMSO); [b]Positive control (Doxorubicin); [c]data significant in relation to control group (vehicle) at $p < 0.001$; **$p < 0.05$ /ANOVA followed by Newman Keuls test.

Table 2. Effect of neosergeolide on HL-60 and PBMC micronucleated cell (MN) frequency in the micronucleus test after 3 h pulse treatment.

	Compound	Treatment (µM)	MN per 1000 BNC[c]	% BNC
HL-60 cells	C[a]	-	9.0 ± 0.57	91.33 ± 0.88
	D[b]	0.6	54.66 ± 2.18*	74.66 ± 1.20*
		0.05	32.33 ± 1.45*	68.33 ± 1.76*
	Neosergeolide	0.1	45.66 ± 1.76*	49.0 ± 1.15*
		0.2	57.33 ± 1.45*	35.0 ± 2.88*
PBMC	C[a]	-	2.50 ± 0.19	88.41 ± 0.33
	D[b]	0.6	46.37 ± 1.25*	63.17 ± 0.18*
		0.05	1.83 ± 0.21	92.16 ± 2.45
	Neosergeolide	0.1	3.25 ± 0.01	84.24 ± 3.00
		0.2	2.10 ± 0.17	87.25 ± 1.15

[a]Negative control (0.1% DMSO); [b]positive control (Doxorubicin); [c]MN frequency is expressed per 1,000 binucleated cells (BNC); *data significant in relation to control group (vehicle) at $p < 0.001$/ANOVA followed by Newman-Keuls test.

Discussion

Previous reports on the cytotoxicity of neosergeolide (Silva et al., 2009) demonstrated that, as observed to other quassinoids (Kupchan et al., 1976; Lee et al., 1982; Lumonadio et al., 1991; Imamura et al., 1993; Mata-Greenwood et al., 2001; Jiang et al., 2008; Lau et al., 2009), it strongly inhibited the proliferation of tumor cells in spite of their histological origin. The present study was designed to evaluate the selectivity of neosergeolide to tumor cells in comparison to normal lymphocytes, and moreover, to elucidate the underlying mechanism of action. MTT analysis confirmed the strong cytotoxicity of neosergeolide to leukemia cells (IC_{50} = 0.1 µM after 24 h of exposure), while it suggest a good selectivity for this compound, since no cytotoxicity was observed to proliferating lymphocytes at tested concentrations. A balance between therapeutic and toxicological effects

of a candidate compound is important for establishing applicability as a pharmaceutical. Also, when considering the side effects of chemotherapy, it is very important to determine whether a drug has a harmful effect on normal dividing cells, such as proliferating lymphocytes (Zucot et al., 2002; Anazetti et al., 2003). Brucein D, a quassinoid isolated from *Brucea javanica* (L.) Merr. (Simaroubaceae), also demonstrated selectivity against pancreatic tumor cells in comparison to nontumorigenic cells (Lau et al., 2009).

In the present study, cytotoxic activity was also evaluated through the loss of membrane integrity as shown by results of flow cytometry analyses, especially after 24 h of exposure. During early stages of apoptosis, cell membrane becomes impermeable to vital dyes, such as trypan blue (Piscentini et al., 1991) or PI (Van Cruchten & Van Den Broeck, 2002), and opposite situation occurs during late apoptosis or necrosis. So, other assays are needed to evaluate cells undergoing early apoptosis (i.e., annexin detection and caspases activation).

Proliferation capacity is equivalent to cell growth and was measured by incorporation of BrdU, a thymidine analog that is incorporated into DNA during the S phase and can be detected by immunocytochemistry (Holm et al., 1998). Over a period of 24 h, neosergeolide at low concentration decreased the number of BrdU-positive cells. The lower BrdU uptake by cell DNA after neosergeolide exposure also corroborates the result obtained from the MTT assay. In addition, our results are in general agreement with the antiproliferative properties of other quassinoids, such as brusatol and bruceantin, which were shown to inhibit the proliferation of several established leukemia cell lines, including HL-60 cells, in an assay based on the incorporation of ^{3}H-thymidine (Mata-Greenwood et al., 2002).

Induction of apoptosis by quassinoids has been reported before (Mata-Greenwood et al., 2002; Cuendet et al., 2004; Rosati et al., 2004; von Bueren et al., 2007; Lau et al., 2009). In this study, several sensitive methods for detecting apoptosis were used, based on the different morphological or biochemical features of apoptosis and necrosis. The results demonstrate that neosergeolide induces apoptosis in HL-60 cells at micromolar concentrations as evidenced by flow cytometric analyses and morphological alterations (May-Grünwald-Giemsa staining). DNA fragmentation during apoptosis could lead to extensive loss of DNA content and a distinct sub-G_0/G_1 peak when analyzed by flow cytometry. In the present study, apoptosis was analyzed by the determination of sub-G_0/G_1 cells. Our analysis revealed that neosergeolide stimulation increased the percentage of sub-G_0/G_1 peaks (hypodiploid DNA) in HL-60 cells in a time- and concentration-dependent manner.

Apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths and can occur simultaneously in tissues and cell cultures exposed to the same stimulus (Nicotera et al., 1999). However, there is no clear biochemical definition of necrotic cell death and consequently no positive biochemical marker that unambiguously discriminate necrosis from apoptosis (late apoptosis features). Another problem is that even the interpretation of dying cell morphology may be complex, because in the absence of phagocytosis apoptotic cells proceed to a stage called secondary necrosis, which shares many features of primary necrosis (Kerr et al., 1994; Kroemer et al., 1998; Krysko et al., 2008).

One of the earliest manifestations of apoptosis, regardless of the initiating stimulus, is the redistribution of phospholipids in the plasma membrane that leads to the exposure of phosphatidylserine (PS) at the cell surface (Fadok et al., 1992; Koopman et al., 1994; Martin et al., 1995). AnnV is a Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS (Krysko et al., 2008). Combining AnnV with PI can help to distinguish between apoptosis (early and late stages) and necrosis. The data showed that after pulse treatment (3 h of neosergeolide exposure following 21 h reincubation period without drug), many cells were in early and late apoptosis with active caspases-3 and -7, and after a short period exposure (3 h), activation of caspases-3 and -7 was also observed. These results showed that the activation of apoptotic mechanisms occurs earlier and do not depend on extended periods of exposure.

When mitochondrial membrane potential is dissipated, a cell begins an irreversible apoptotic process (Gao et al., 2006; Hsu & Yen, 2007). Detection of mitochondrial membrane potential changes can, therefore, be useful as a probe for the onset of apoptosis. The correlation between the loss of the mitochondrial membrane potential and DNA fragmentation indicates that the reduction of $\Delta\psi_m$ constitutes an obligate and irreversible step of ongoing HL-60 death. In this study, neosergeolide increased potential loss in the mitochondrial membrane thus providing evidence for the activation of an intrinsic apoptosis pathway in HL-60 cells. These results are in general agreement with the findings of Rosati et al. (2004) who demonstrated that quassinoids induce mitochondrial depolarization and caspase-3 activation. Also, according to Mata-Greenwood et al. (2002), treatment of leukemic cells with bruceantin induces a decrease in *c-myc* mRNA and protein which in turn may be responsible for some of the pro-apoptotic effects of quassinoids (Cuendet & Pezzuto, 2004).

Mitochondria play a decisive role in the apoptotic pathway mediated by certain agonists (Green, 1998; Reed et al., 1998; Smaili et al., 2003). Disruption of the inner and outer mitochondrial membrane and opening of the MPT pore, which is regulated by members of the Bcl-2 family as well as the redox and energy state of the cell, result in a collapse of $\Delta\psi_m$, and in the exit of soluble proteins, such as cytochrome *c* and apoptosis-inducing factor (van Loo et al., 2002). This MPT from the intermembrane space can trigger an activation of downstream caspases (Zoratti & Szabo, 1995; Thornberry &

Lazebnik, 1998). The classic MPT is dependent upon the $[Ca^{2+}]_i$, is energy dependent, and is accompanied by mitochondrial swelling and depolarization (Zoratti & Szabo, 1995), which CsA inhibits MPT pore opening (Armstrong, 2006). The CsA binds to Cyp-M, a cyclophilin-family protein associated with the MPT pore, causing it to dissociate from the pore complex, and this increases the probability of MPT pore closure and thus prevents the $\Delta\psi_m$ disruption and block cytochrome *c* release (Lemasters, 1999; Jiang et al., 2001; Brustovetsky et al., 2002). On the contrary, it has been reported that the nonclassic MPT, which is insensitive to CsA, occurs without swelling and depolarization of the mitochondria (Sultan & Sokolove, 2001).

Consistent with these observations, neosergeolide-induced apoptosis is dependent of the classic MPT mechanism. Cotreatment with CsA prevents neosergeolide-induced caspases-9, -3 and -7 activation suggesting that blocking of MPT prevents the leakage of cytochrome *c* and consequently prevents the activation of caspase-9 (caspase-dependent cytochrome *c* release) and apoptotic protease activating factor 1 (Apaf-1). Caspase-9 is thought to be the initiator caspase involved in the mitochondrial-initiated apoptotic pathway, and it activates downstream caspases, such as caspases-3, -6, and -7 (Strasser et al., 2000). Caspases-3 and -7 are two well-known "executioner caspases." Their activation is believed to be responsible for the morphological changes seen in apoptosis, including DNA fragmentation, chromatin condensation, and the formation of apoptotic bodies (Marcelli et al., 1999; Nicholson, 1999).

Several mechanisms exist by which neosergeolide could potentially exert to achieve the observed apoptogenic effects in cancer cells. It was evaluated whether the ROS generation or direct DNA damage could be related to neosergeolide activity. Apoptosis induced by many chemical genotoxins is a consequence of blockage of DNA replication, which leads to collapse of replication forks and DNA double-strand breaks formation, which, the latter, is thought to be crucial downstream for apoptosis-triggering lesions (Roos & Kaina, 2006). Genotoxic DNA damaging agents may activate both membrane death receptors and the endogenous mitochondrial damage pathway leading to cell death via apoptosis (Kaina, 2003). DNA damage, such as DNA strand breakage and induction of MN, may be important features of neosergeolide's cytotoxic mechanisms. Thus, HL-60 cultures treated with neosergeolide exhibit strong reduction in cytokinesis-block proliferation which is a biological parameter for the detection of cellular toxicity or cell cycle delay (Surrallés et al., 1995). Also, neosergeolide induces DNA damage after a short incubation period (3 h) as evidenced by a significant increase in grades 3 and 4 comets in comparison to negative control (Figure 7 A). The occurrence of comets with no heads and with nearly all DNA in the tail (grade 4) is an indication of the cytotoxic effect (Hartmann & Speit, 1997). Interestingly, neosergeolide induces DNA damages in HL-60 cells which are p53 null (Shimizu & Pommier, 1997) but not in PBMC (wild-type p53), suggesting that this effect is p53 independent. The tumor suppressor protein p53 is considered to be a major player in the apoptotic response to genotoxins. Some experiments, trying to elucidate in more detail the role of p53 in DNA damage-triggered apoptosis, have shown that some primary and established cell (mouse fibroblasts) lines deficient for p53 were clearly more sensitive than the corresponding wild-type after exposure to UV-C and alkylating agent (methyl methanesulfonate), supporting the view that p53 is not required for inducing apoptosis in these cells (Lackinger & Kaina, 2000; Lackinger et al., 2001). However, the pro- or anti-apoptotic effect of p53 appears to be a cell type-specific phenomenon since lymphoblastoid cells wild-type for p53 proved to be more sensitive to alkylating agents and UV-C than the p53 mutated counterparts (Karran & Stephenson, 1990). The factor(s) involved in making the decision between protection against or stimulation of the apoptotic process by p53 remains unknown (Kaina, 2003).

Intracellular ROS production is associated with a number of cellular events, including activation of NADH oxidase and xanthine oxidase, and the functioning of the mitochondrial respiratory chain (Perez-Ortiz et al., 2007). The NADH oxidase is inhibited by several known potential antitumor agents, such as sulfonylurea, adriamycin, and capsaicin (del Castillo-Olivares et al., 1998). Interestingly, Morré et al. (1998) showed that the cytotoxicity of the quassinoid glaucarubolone to HeLa cells was associated with NADH oxidase inhibition. Zhao et al. (2008) demonstrated that inhibition of NADPH oxidase activity by diphenyleneiodonium suppressed free radical production and inhibited cell growth of B16 melanoma cells. The present study provides evidence that the mechanisms of cell growth inhibition, cell death, and DNA-damage of neosergeolide do not depend on the production of ROS. These data are consistent with the previous report in which it was shown that the cytotoxicity of neosergeolide and another isolated quassinoid (isobrucein B) toward cancer cell lines is not related to oxidative stress (Silva et al., 2009). A working mechanistic model is developed based on these findings and is summarized in a schematic diagram (Figure 8).

Conclusions

The present study provided experimental evidence to support the underlying mechanism of action involved in the neosergeolide-mediated apoptosis. Taken together, the results indicated that neosergeolide leads to DNA damage triggering intrinsic pathways for apoptosis induction. In addition, no antiproliferative effect or DNA damage effect of neosergeolide was evident in PBMC, which is evidence of its therapeutic potential.

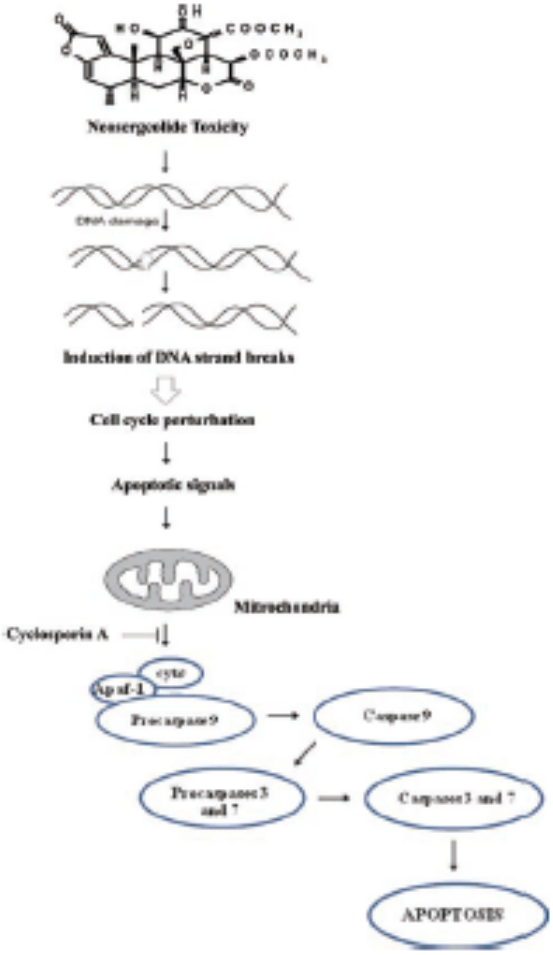

Figure 8. Summarized model of neosergeolide antiproliferative effects.

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Declaration of interest

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CAPÍTULO 7: NOVAS CONTRIBUIÇÕES PARA O CONHECIMENTO DA COMPOSIÇÃO QUÍMICA E ATIVIDADE BIOLÓGICA DE INFUSÕES, EXTRATOS E QUASSINÓIDES OBTIDOS DE *Picrolemma sprucei* Hook.f. (SIMAROUBACEAE).

1. Material e Métodos

1.1 - Coleta e identificação do material vegetal

A espécie *Picrolemma sprucei* foi coletada em dois momentos: Uma primeira coleta foi efetuada pelo Dr. Adrian M. Pohlit no município de Silves, Estado do Amazonas, em abril de 2003. Exsicatas foram depositadas no Herbário da UFAM (Silva 5729 e Silva 5730). A identificação foi realizada pelo Dr. Wayt Thomas (comunicação pessoal). Uma segunda coleta foi realizada na Reserva Florestal ZF-2 (km 60 BR-174, Manaus - Boa Vista) pertencente ao INPA, em setembro de 2006. Foi feita uma triagem das plantas, separando-as em raízes, caules, folhas e frutos. A confirmação da identificação botânica da espécie *P. sprucei* foi realizada no Herbário do Instituto Nacional de Pesquisa da Amazônia (INPA), onde foi depositada amostra fértil (exsicata) referente a essa espécie (223883). As distintas partes da planta foram secas à sombra, picotadas em pedaços de cerca de 10 cm. Os caules e raízes foram moídos em moinho "Whiley Mill" n^{o} 3, marca Arthur H. Thomas (Philadelphia, U.S.A.), e os frutos foram moídos em moinho de carne, marca Eberle.

1.2 - Reagentes e solventes

Todos os solventes utilizados neste trabalho foram de grau técnico e previamente purificados utilizando destilação fracionada. Os solventes foram tratados para a retirada de impurezas comuns e secos com Na_2SO_4 quando necessário. A água utilizada em todas as etapas foi destilada.

1.3 - Equipamentos

Foram utilizados os seguintes equipamentos: Balança semi-analítica, Marca Quimis, modelo BG2000, limite 2,020g, balança analítica, Marca Mettler-Toledo, modelo AB204 com limite máximo de peso de 210 mg, evaporador rotatório, Marca Fisatom 802, modelo 550, lâmpada UV, Marca Spectroline, modelo CX-20, "ultraviolet fluorescence analysis cabinet", onda longa (365 nm) e onda curta (254 nm), ultra-som, Marca Ultrasonic, modelo USC 1400.

1.4 - Análises cromatográficas

1.4.1 - Cromatografia de camada delgada (CCD) analítica

Foram utilizadas placas prontas de sílica gel 60 em alumínio MERCK de 20 x 20 cm com indicador de fluorescência F_{254}. Os sistemas de revelação utilizados foram lâmpadas de ultravioleta (UV) a 254 nm e 366 nm e revelação em vapor de iodo e solução alcoólica de H_2SO_4 a 5% e vanilina. Para análise em fase reversa foram utilizadas cromatoplacas de RP-18 em alumínio MERCK de 20 x 20 cm com indicador de fluorescência F_{254}, utilizando-se os mesmos sistemas de revelação.

1.4.2 - Cromatografia de camada delgada (CCD) preparativa

Foram utilizadas placas com sílica gel 60 em alumínio MERCK com 1 mm de espessura e tamanho de placas 20 x 20 cm. Para revelação serão utilizadas lâmpadas de UV a 254 e 366 nm.

1.4.3 - Cromatografia Líquida de Alta Eficiência (CLAE)

Cromatógrafo analítico Shimadzu, modelo LC-20, com sistema quaternário, detector SPD-20A, injetor automático SIL-20A e controladora CBM-20A. A fase móvel utilizada foi acetonitrila-água 10:90, com gradiente linear (20 min) até acetonitrila-água 75:25 e isocrático até o final da corrida. Foi utilizada coluna de fase reversa analítica LichroCart® 250-4 com volume de injeção de 10 μL, fluxo de 1,5 mL/min.

1.5 - Análises espectroscópicas

Espectrômetro de ressonância magnética nuclear Marca Varian, modelo Unity Inova (500 MHz) do Centro de Biotecnologia da Amazônia (CBA). Solventes: $CDCL_3$, MeOD, D_2O e C_5D_5N. Tetrametilsilano (TMS) foi utilizado como padrão interno.

Espectrômetros de massas Daltronics-Bruker UltrOtof Mass Spectrometer e Daltronics-Bruker Ion Trap Mass Spectrometer (USP-SP, Central Analitica). Espectros de massa acurada por ionização de "electrospray" em modo positivo foram adquiridos em um aparelho Bruker-Daltronics UltrOTof utilizando uma mistura de $MeOH/H_2O/HCO_2H$ como sistema de solventes para infusão [infusão significa que a substancia foi dissolvida numa mistura desses solventes e a solução resultante foi injetada diretamente no aparelho de massas.

1.6 - Obtenção dos quassinóides

A preparação dos extratos de *P. sprucei* foi realizada por Silva (2006), descrita no **capítulo 1.**

Novo método: isolamento da neosergeolida a partir de raízes em duas etapas

Dando seqüência ao aperfeiçoamento do procedimento de extração dos quassinóides, 450 g de serragem de raízes, obtidas na segunda coleta, foram submetidas a desgraxificação com hexano (1,0 L) em extratores do tipo Soxhlet (3 x 6 h). O solvente foi evaporado utilizando-se evaporação rotatória obtendo-se o extrato em hexano (massa?). A serragem foi submetida à extração com Et_2O etílico (1,0 L) a temperatura ambiente (3 x uma semana). O solvente foi evaporado utilizando-se evaporação rotatória originando 2,7 g (0,6%) de extrato em Et_2O denominado PSE, o qual foi submetido à recristalização da forma descrita anteriormente, obtendo-se 219,1 mg de neosergeolida (**5**) (0,049 % em relação ao peso seco de serragem).

1.7 - Preparação dos derivados semi-sintéticos a partir da neosergeolida e isobruceína B

1.7.1 - Hidrólise alcalina

A reação de hidrólise alcalina foi realizada segundo o procedimento descrito por Okano e colaboradores (1985) que aplicaram essa reação a isobruceína B (**2**). A uma solução de isobruceína B (**2**) (25,4 mg) em MeOH (1,5 mL) foi adicionado NaOH 1 N (0,45 mL). Após agitação a -5 ^{o}C por 17 min, a mistura de reação foi neutralizada com HCl 1N (0,8

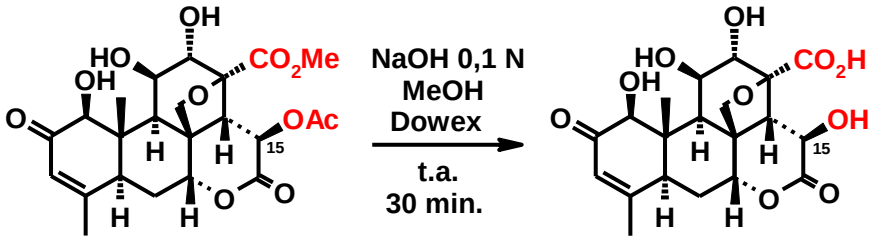
mL) e evaporada a vácuo para obtenção de um resíduo branco cristalino. A reação foi monitorada por CCD analítica como descrito anteriormente. O resíduo foi submetido à purificação em cromatografia em coluna de sílica Flash (MeOH, 100%) para obtenção de 15,6 mg do derivado **23** (Esquema 1), na forma de um cristal branco (Rf 0,38; Tempo de retenção CLAE 5'48"; UVmáx 245nm). Da mesma forma, 13,1 mg do derivado desacetilado **24** (Esquema 1), na forma de um cristal branco (Rf 0,51; Tempo de retenção CLAE 8'27"; UVmáx 245nm) foram preparados a partir de neosergeolida (**5**) (25,3 mg). Na figura 1 observa-se a ampliação dos espectros de ^{1}H de **5** e **24**. No quadro A observa-se o sinal em 2.9 ppm referente ao grupo acetil ligado ao carbono 15. No quadro B observa-se claramente a ausência deste sinal.

Outro procedimento de hidrólise alcalina foi realizado adicionando-se NaOH 0,1 N (5 mL) a uma solução do quassinóide (16,1 mg) em MeOH (10 mL). Após agitação a -5 ^{o}C por 30 min, a mistura de reação foi neutralizada com resina Dowex, filtrada e evaporada a vácuo para obtenção de um resíduo branco cristalino. A reação foi monitorada por CCD analítica como descrito anteriormente. O resíduo foi submetido à purificação em cromatografia em coluna de sílica Flash, com eluição em i-PrOH, para obtenção de 12,4 mg do derivado **25** (Figura 2) (MS/MS = 425,1409 [M + H]$^{+}$; 447,1238 [M + Na]$^{+}$, na forma de um cristal branco.

1.7.2 - Redução

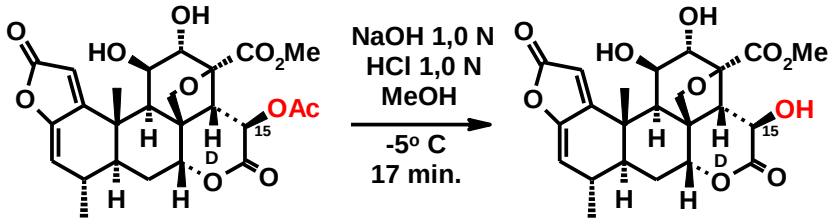
A uma solução de neosergeolida (**5**) (34,1 mg) em MeOH (10,0 mL) foi adicionado $NaBH_4$ (1,0 mg). A solução ficou sob agitação por 24 h. Após purificação por CC (MeOH:H_2O, 1:1 v/v) obteve-se o derivado **35** (33,7 mg). Da mesma forma, foram

preparados 33,7 mg do derivado **36** (Rf 0,6; Tempo de retenção CLAE 9’52”; UVmáx 276nm; MS/MS 449,3 $[M - H]^+$ (Figura 3)).



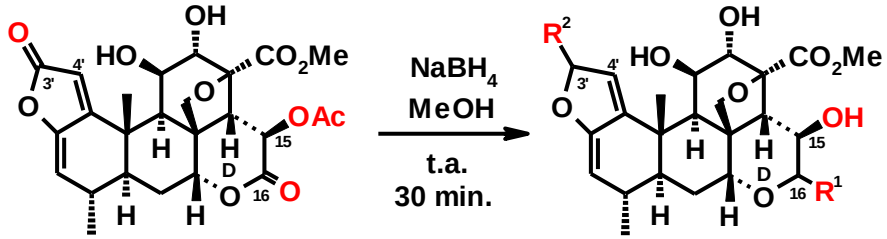
(2)
Isobruceína B

(25)
15-desacetil isobruceína B



(5)
Neosergeolida

(24)
15-desacetilneosergeolida



(5)
Neosergeolida
(34,1 mg)

(36a), R^1 = —OH; R^2 = =O
(36b), R^1 = =O ; R^2 = —OH
(33,7 mg)

Esquema 1 – Preparo de derivados semi-sintéticos a partir dos quassinóides neosergeolida e isobruceína B.

1.8 - Determinação das estruturas cristalográficas

Para as determinações estruturais dos cristais obtidos por difração de raios-X foi utilizado o equipamento modelo URD6, da Carl Zeiss, do Instituto de Química da USP de São Carlos, pelas Professoras Regina H. de A. Santos (USP-SC) e Cláudia C. Silva (UEA). O tratamento dos dados coletados foi realizado no sistema WinGX (FARRUGIA, 1999). Foram realizadas correções pelos fatores de Lorentz, de polarização e dos efeitos de absorção. A estrutura foi resolvida pelos Métodos Diretos através do sistema SIR97 (ALTOMARE et al., 1999), onde foram localizados todos os átomos, menos os hidrogênios. O refinamento foi realizado pelo método dos mínimos quadrados com matriz completa e cálculos de Fourier diferença utilizando o programa SHELXL-97 (HERBST-IRMER; SHELDRICK, 1998).

1.9 - Estudo fitoquímico dos frutos de *P. sprucei*

1.9.1 - Preparação do extrato etanólico dos frutos

Frutos de P. sprucei foram coletados a partir de espécimes cultivados no INPA em dezembro de 2005. 191,26 g de frutos secos em estufa a 50º C e moídos com grau e pistilo foram submetidos à extração em etanol a t.a. por uma semana em um erlenmeyer. Após filtragem em papel de filtro, o extrato foi concentrado utilizando evaporação rotatória. O *marc* foi extraído mais duas vezes e os extratos foram reunidos totalizando 22,97 g (12,0%) de extrato etanólico denominado 1RA10. O mesmo procedimento foi realizado com 16,29 g da casca e polpa dos frutos obtendo-se 1,53 g de extrato etanólico das cascas denominado

3RA16C (8,2%) e com 18,69 g do caroço dos frutos obtendo-se 0,94 g de extrato etanólico da polpa denominado 3RA16P (5,8%).

1.9.1 - Fracionamento do extrato etanólico dos frutos inteiros, cascas e caroços

O extrato etanólico dos frutos inteiros de *P. sprucei* (1RA10) (20,0 g) foi submetido à partição em solução de 100 mL de MeOH/H_2O (9:1) e extraído com 50 mL de hexano por três vezes. As fases hexânicas foram reunidas, secas por evaporação rotatória, obtendo-se 224,8 mg (10,95%) da fase hexânica denominada 3RA22H. Houve a formação de um precipitado branco (3RA22HC) que foi separado e cristalizado. A fase hidrometanólica resultante foi submetida ao mesmo procedimento, substituindo-se seqüencialmente hexano por clorofórmio, acetato de etila e n-butanol (Figura 5). Os extratos etanólicos das cascas (3RA16C) e caroços (3RA16P) (0,5 g de cada) também foram submetidos a procedimentos de partição como descrito acima (Figuras 6 e 7).

1.10 - Ensaios biológicos

1.10.1 - Ensaio de toxicidade frente a larvas de *A. franciscana*

O ensaio de letalidade frente a larvas do microcrustáceo *A. franciscana* Kellogg *in vivo* foi realizado no LAPAAM/INPA segundo procedimentos descritos no capítulo 2 desta Tese.

1.10.2 - Avaliação da atividade inseticida frente a imagos de *A. aegypti*

Essa atividade foi determinada por prova biológica de garrafas de 250 mL Marca *Schott* segundo protocolo de WHO (2006), onde foram usados mosquitos adultos hematófagos da espécie *Aedes aegypti*, com 3 a 5 dias de idade, obtidos de campo. As substâncias de interesse foram impregnadas nas garrafas e colocadas em contato com os mosquitos. O teste foi realizado em triplicata fazendo-se observações a cada 10 min até 90 min. O resultado foi avaliado pelo percentual de mortalidade até 90 min, onde o total de mosquitos mortos foi dividido pelo total de mosquito inicial e multiplicando-se por 100.

1.10.3 - Avaliação da atividade antiproliferativa em células tumorais

Os ensaios de citotoxicidade frente a células tumorais *in vitro* foram realizados no Laboratório de Oncologia Experimental (LOE) do Departamento de Fisiologia e Farmacologia da Universidade Federal do Ceará (UFC), sob coordenação da Professora Cláudia Pessoa, com colaboração do aluno de Doutorado em Farmacologia Bruno Coelho Cavalcanti, segundo procedimento descrito no Capítulo 2 desta Tese.

1.10.5. Ensaio de inibição da atividade de mitocôndrias vegetais.

Os ensaios de inibição da atividade mitocondrial foram realizados no Laboratório de Bioenergética do Departamento de Bioquímica e Biologia Molecular da UFC, sob coordenação da Professora Dirce Fernandes. As mitocôndrias foram isoladas de raízes do cultivar de feijão Vita 5 em condições controle pelo método de Cornu e colaboradores

(1996). A concentração de proteína mitocondrial foi determinada pelo método de Bradford (1976). A análise da integridade da membrana mitocondrial externa foi avaliada segundo método descrito por Neuburger e colaboradores (1982).

1.10.6. Ensaio de atividade hemolítica em eritrócitos de camundongos *Mus musculus* Swiss

Os ensaios de atividade hemolítica foram realizados no Laboratório de Oncologia Experimental (LOE) do Departamento de Fisiologia e Farmacologia da Universidade Federal do Ceará (UFC), sob coordenação da Professora Cláudia Pessoa, com colaboração do aluno de Doutorado em Farmacologia Bruno Coelho Cavalcanti. Os ensaios de atividade hemolítica foram realizados no LOE-UFC, segundo a metodologia descrita por Costa-Lotufo e colaboradores (2002) e Dresch e colaboradores (2005). Essa metodologia permite avaliar o potencial das substâncias-testes em causar lesões na membrana plasmática dos eritrócitos, seja pela formação de poros ou pela ruptura total. O sangue foi coletado de três camundongos (*Mus musculus* Swiss) por via orbital (animais previamente anestesiados com éter etílico) e diluído na proporção de 1:30 com solução salina (NaCl 0,85% + CaCl2 10 mM). Os eritrócitos foram lavados 2 vezes em solução salina por centrifugação (15 g/3 min.) para redução da contaminação plasmática e ressuspensos em solução salina para obtenção de uma suspensão de eritrócitos (SE) a 2%. Os ensaios foram realizados em multiplacas com 96 cavidades. Cada poço da 1ª fileira recebeu 100 µL da solução salina. Na 2ª, os poços receberam 50 µL da solução salina e 50 µL do veículo de diluição da substância teste, neste caso, DMSO 10%. Aos poços da 3ª fileira, foram adicionados 100 µL de solução salina e 100 µL das substâncias teste em solução. Da 4ª fileira em diante os

poços receberam 100 µL da solução salina, excetuando-se os da última fileira, que receberam 80 µL de solução salina e 20 µL de Triton X-100 a 1% (controle positivo). As diluições foram feitas da 3ª à 11ª cavidade, retirando-se 100 µL da solução da cavidade anterior e transferindo para a seguinte de modo que as concentrações foram sempre diluídas pela metade, variando de 1,5 a 200 µg/mL. Em seguida, 100 µL da suspensão de eritrócitos foram plaqueados em todos os poços. Após incubação de 1 hora, sob agitação constante à temperatura ambiente (26 ± 2ºC), as amostras foram centrifugadas (50 g/3 min.) e o sobrenadante transferido para uma outra placa para a leitura da absorbância da hemoglobina no espectrofotômetro de placas a 540 nm. A atividade das substâncias teste foi determinada de maneira relativa ao valor dos controles positivo e negativo.

1.10.7. Cultura de parasitas e ensaio de atividade antimalárica *in vitro*

1.10.7.1. Técnica de microscopia

Os ensaios de atividade antimalárica foram realizados por este Doutorando no Setor de Malária da Fundação de Medicina Tropical do Amazonas (FMTAM), sob orientação do Dr. Pedro P. R. Vieira, segundo metodologia descrita no Capítulo 2 desta Tese.

1.10.9. Ensaios de atividade antibacteriana segundo o teste de difusão em ágar

Os testes de atividade antibacteriana dos quassinóides foram realizados no laboratório do Professor Denilson F. de Oliveira da Universidade Federal de Lavras (UFLA). Foram utilizadas linhagems das bactérias *Aeromonas hydrophila* (ATCC 7966), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 25853) e *Staphylococcus aureus*

(ATCC25923). As bactérias foram repicadas para placa de Petri contendo meio TSA e, após 24 horas, foram transferidas para tubo de solução aquosa de NaCl a 0,85 % (g/mL), até se alcançar turvação 0,5 na escala de Mc Farland (1,0 para *B. subtilus*). Com um *Swab* estéril, as bactérias contidas nessas soluções salinas foram inoculadas em meio de cultura Muller-Hinton-Agar contido em placas de Petri. A seguir, foram feitos furos de 0,6 cm de diâmetro nos meios de cultura inoculados, nos quais foram depositados 40 µL das soluções resultantes da dissolução dos extratos (5 mg) em 1 mL de etanol/água (7:3). Após 24 horas a 37 ^{o}C, os halos de inibição do crescimento bacteriano ao redor do furo foram medidos com uma régua. Os experimentos foram realizados em duplicata, com norfloxacina (ou cloranfenicol) e etanol/água como controles positivo e negativo, respectivamente.

1.10.10. Ensaios de atividade antifúngica

Os testes de atividade antifúngica dos quassinóides foram realizados no laboratório do Professor Denilson F. de Oliveira (UFLA). Foi utilizada a espécie de fungo *Aspergillus ochraceus* Wilhem. 1,0 mg dos quassinóides foram dissolvidos em 2,0 mL de Tween 80 a 1% (g/mL) e adicionou-se 200 microlitros das soluções resultantes a 40 microlitros de uma suspensão aquosa contendo 8,0x10^{8} esporos/mL do fungo *Aspergillus ochraceus* Wilhem. O meio de cultura Czapeck Yeast Extract (CYA-200 microlitros), ainda quente, foi adicionado à cavidades de placas de 96 cavidades (capacidade aproximada de 400 microlitros por cavidade) e, após solidificação do meio, 20 microlitros das suspensões de esporos contendo extratos vegetais foram colocados em cada furo. Após 48 horas na temperatura de 25° C, o experimento foi avaliado por observação visual para verificar se houve crescimento fúngico ou não. Os experimentos foram realizados em duplicata,

empregando-se Tween 80 a 1% e cloreto de benzalcônio como controles negativo e positivo (MOURI et al., 2005).

1.10.11. Ensaios de atividade anti-helmíntica

Os testes de atividade anti-helmíntica dos quassinóides foram realizados no laboratório laboratório do Professor Denilson F. de Oliveira (UFLA). Raízes de cafeeiros (*Coffea arabica* L.) cultivados em casa de vegetação e infestadas com *Meloidogyne exigua* Goeldi, foram lavadas cuidadosamente e cortadas em pedaços de um centímetro. A seguir, foram trituradas em liquidificador por vinte segundos em solução de hipocloprito de sódio a 0,5 %, seguindo a técnica de Hussey e Barkey (1973), modificada por Boneti & Ferraz (1981). Os ovos retidos na peneira de 0,025 mm foram recolhidos em béquer de 200 mL, utilizando-se pisseta contendo água destilada. Para a obtenção dos juvenis dos segundo estádio (J2), utilizou-se uma câmara de eclosão formada com tela e papel de espessura fina, colocado num funil de vidro esterilizado. Apenas J2 com no máximo dois dias de idade após eclosão foram empregados. Em cada cavidade de placas do tipo Elisa foram colocados 100 µL de solução aquosa de Tween 80 a 1% contendo os quassinóides neosergeolida e isobruceína b (500 ppm) e 20 µL de suspensão aquosa contendo de 20 a 30 J2. Após 48 horas no escuro, a 25^{o}C, contou-se os indivíduos móveis e imóveis. A seguir, contou-se os mortos e vivos. O experimento foi realizado com seis repetições e como testemunha utilizou-se Tween 80 a 1%.

1.11. Análises Estatísticas

Os dados estão apresentados na forma de médias ± desvios padrões. Os valores de concentração inibitória 50 (CI_{50}) ou concentração efetiva 50 (CE_{50}) e seus intervalos de confiança 95% (IC95%) foram obtidos por regressão linear utilizando o programa GRAPHPAD (Intuitive Software for Science, San Diego, CA). Os valores de concentração letal 50 (CL_{50}) para os testes de letalidade de larvas de *A. franciscana* foram obtidos de contagens de larvas vivas após 24 h de incubação com as substâncias testadas vida, utilizando-se o método de análise de probito descrito por Finney (1971).

2. Resultados e Discussões

2.1 - Coleta e identificação do material vegetal

Foram obtidos 43 acessos de *P. sprucei* na segunda coleta efetuada na Reserva ZF2. Uma nova exsicata apresentando frutos está em fase de preparo. Os dados referentes a massa úmida e seca podem ser observados na Tabela 1.

Tabela 1 - Dados da perda de umidade de *P. sprucei* coletada na Reserva ZF2.

Órgão	Peso fresco (g)	Peso seco na estufa (g)	Massa seca (%)
Raízes	7.810,73	4.174,38	53,44
Caules	2.145,88	1.042,59	48,59

2.2 - Obtenção dos quassinóides

Moretti e colaboradores (1982) utilizaram de forma exaustiva, desgraxificações, seguidas de infusões seriadas com água quente, extração líquido-líquido com água e clorofórmio e cromatografias as quais correspondem a um processo lento, especialmente em uma escala piloto. Na adaptação iniciada por Silva (2006) e concluída no presente trabalho, o uso de extração contínua líquido-sólido gerou extratos aquosos parcialmente concentrados e ricos em quassinóides. Enquanto Moretti e colaboradores (1982) geraram um extrato em $CHCl_3$, que foi cromatografado para obtenção de (**5)** e (**2)** em escala de miligramas, o

método desenvolvido por Silva (2006) eliminou completamente o uso de procedimentos cromatográficos. A etapa chave de precipitação removeu substâncias muito polares e solúveis em água, presentes no extrato obtido em clorofórmio e permitiu a recristalização diferencial de isobruceína B (**2**), 1,17 g, com rendimento de 0,018% em relação ao peso seco da serragem e 0,23% em relação ao peso seco do extrato e neosergeolida (**5**), 1,75 g, com rendimento de 0,02% em relação ao peso seco de serragem e de 0,34% em relação ao peso seco do extrato.

Aqui estamos demonstrando a seqüência do processo de melhoramento da extração dos quassinóides em escala-grama, a temperatura ambiente e com solventes não clorados, seguido de recristalização. Até o momento foram obtidos seletivamente 219,1 mg de neosergeolida (**5**) (0,049 % em relação ao peso seco de serragem), aproximadamente o dobro em relação ao procedimento descrito por Silva (2006). Esta nova tentativa de extração e isolamento dos quassinóides de *P. sprucei* ainda se encontra em estudo a fim de determinarmos um procedimento ideal para obtenção da isobruceína B (**2**). Vale ressaltar ainda o encurtamento do tempo necessário para obtenção da neosergeolida (**5**) de vários meses para poucas semanas.

O Laboratório de Pesquisas em Produtos Naturais (LAPAAM) já possui grande experiência no estudo dos quassinóides de *P. sprucei*, já tendo sido produzidos duas dissertações e quatro artigos referentes aos estudos da isobruceína B (**2**) e neosergeolida (**5**) (Saraiva, 2001, Silva, 2006). Silva (2006) descreveu ainda a preparação dos derivados acetilados **21** e **22** bem como iniciou o estudo das atividades citotóxica e larvicida dessas substâncias naturais e semi-sintéticas. Dessa forma, não serão apresentados aqui dados espectroscópicos das substâncias (**2**), (**5**), **21** e **22** por já se tratarem de dados largamente descritos e estudados pelo grupo.

Os dados da análise de difração de raios-X (Figura 8) confirmaram os dados previamente descritos na literatura nos quais a neosergeolida (**5**) forma cristais com água de cristalização (Zukerman-Schpector et al., 1994). Todas as substâncias geradas neste trabalho e que se apresentam na forma de cristal, também estão sendo analisadas segundo esta técnica.

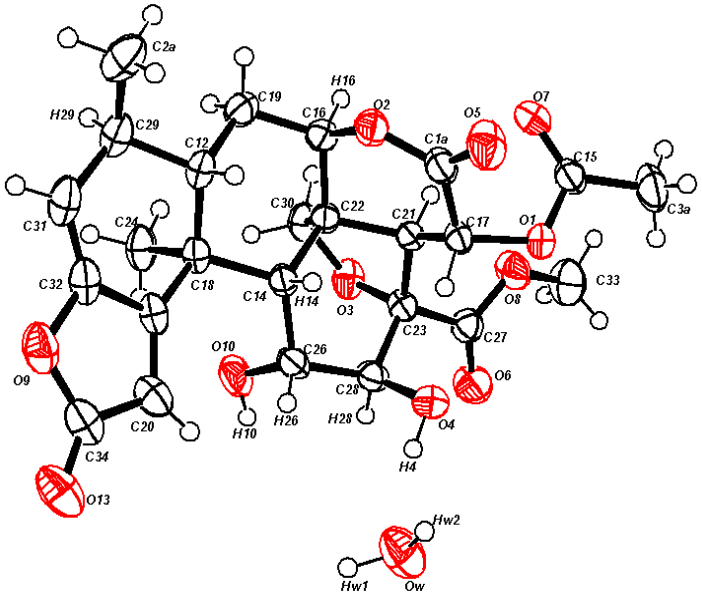


Figura 8. Representação ORTEP da estrutura **5,** identificada por difração de raios-X de monocristais.

2.3 - Preparação dos derivados semi-sintéticos a partir da neosergeolida e isobruceína B

4.3.1 - Hidrólise alcalina

O derivado **23**, obtido por reação de hidrólise alcalina da isobruceína B (**2**), apresentou fator de retenção (r.f.) de 0,38 mm por CCD analítica (Et_2O:i-PrOH, 9:1) e tempo de

retenção de 5 min e 40 seg. por CLAE e teor de pureza do pico maior do que 98%. A absorção máxima no UV (UVmáx.) foi de 245 nm. Já o derivado **24**, obtido por reação de hidrólise alcalina da neosergeolida (**5**) apresentou r.f. de 0,51 mm, tempo de retenção de 8 min e 27 seg. e teor de pureza do pico maior do que 98%.

O derivado **25**, obtido por reação de hidrólise alcalina da isobruceína B (**2**), apresentou fator de retenção (r.f.) de 0,29 mm por CCD analítica e tempo de retenção de 1 min e 44 seg. por CLAE, teor de pureza do pico maior do que 98%, com UVmáx de 245 nm. O espectro de massas obtido (ESI-MS, CH_3OH:H_2O:HCO_2H, Figura 10) apresentou fragmentações (m/z) em 425,1409 $[M + H]^+$ e 447,1238 $[M + Na]^+$ compatíveis com a estrutura proposta para o derivado **25** (Figura 10).

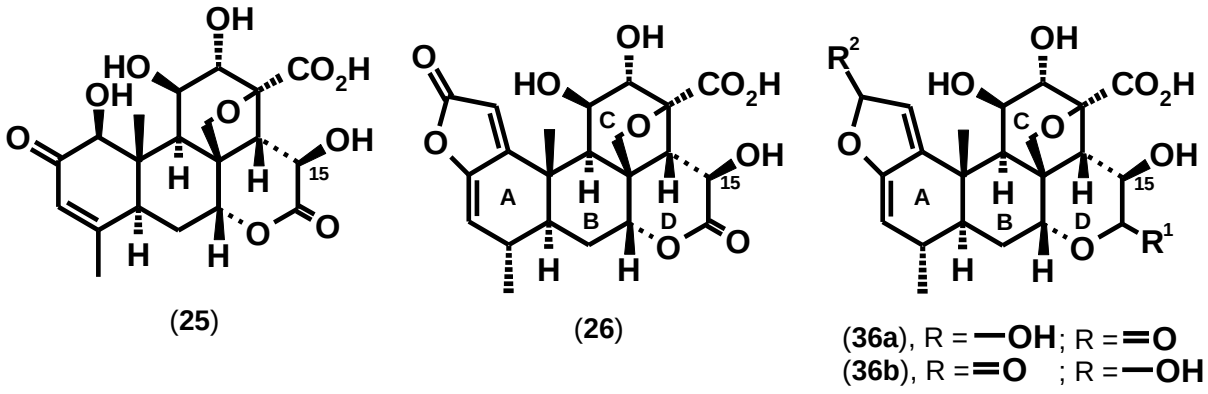


Figura 10. Proposta estrutural para os quassinóides semi-sintéticos preparados a partir da neosergeolida e isobruceína B.

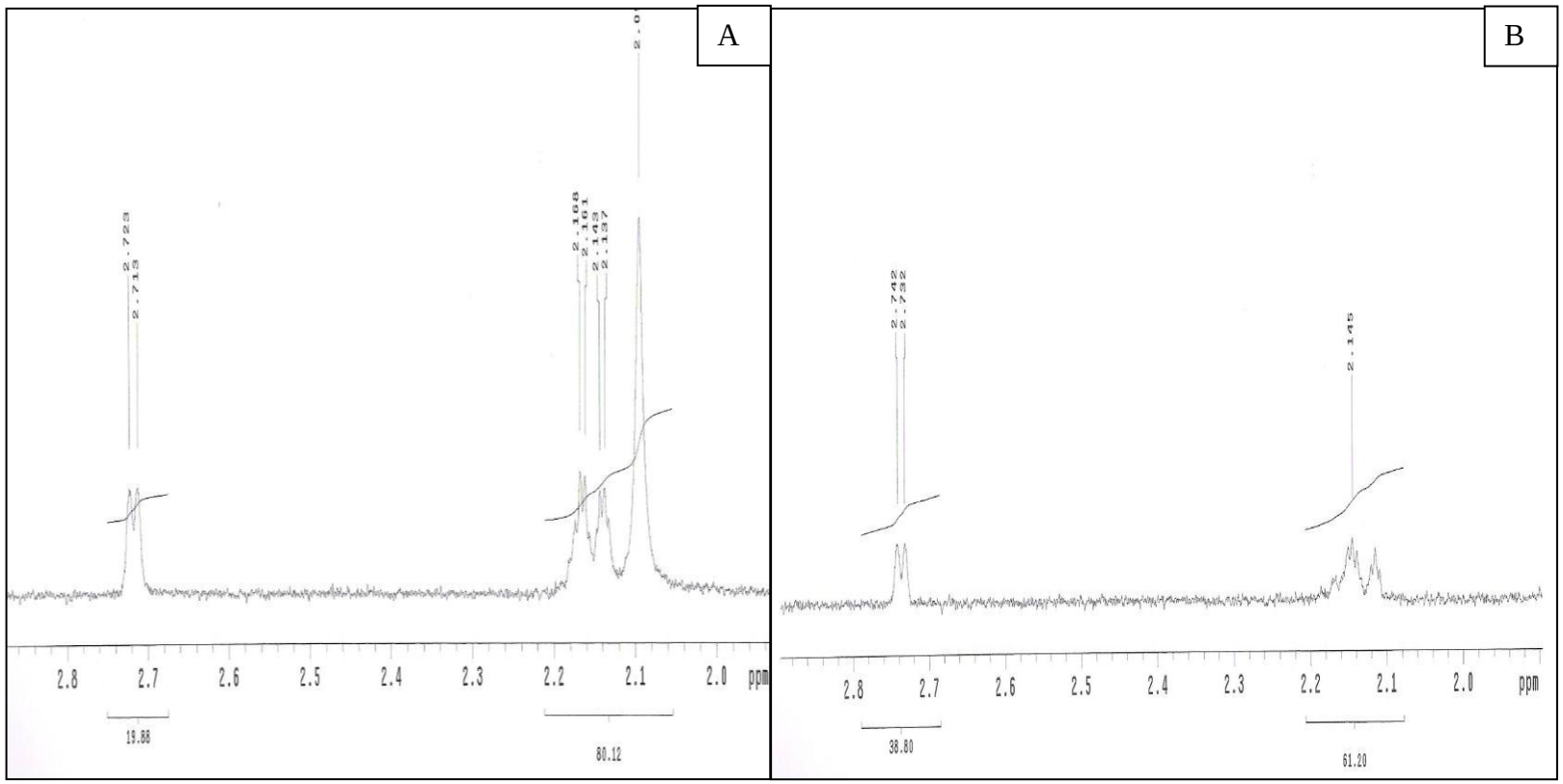


Figura 9 – Ampliação dos espectros de RMN ^{1}H da neosergeolida (**5**) (A) e seu derivado desacetilado (**24**) (B) ($CDCl_3$ / 500MHz)

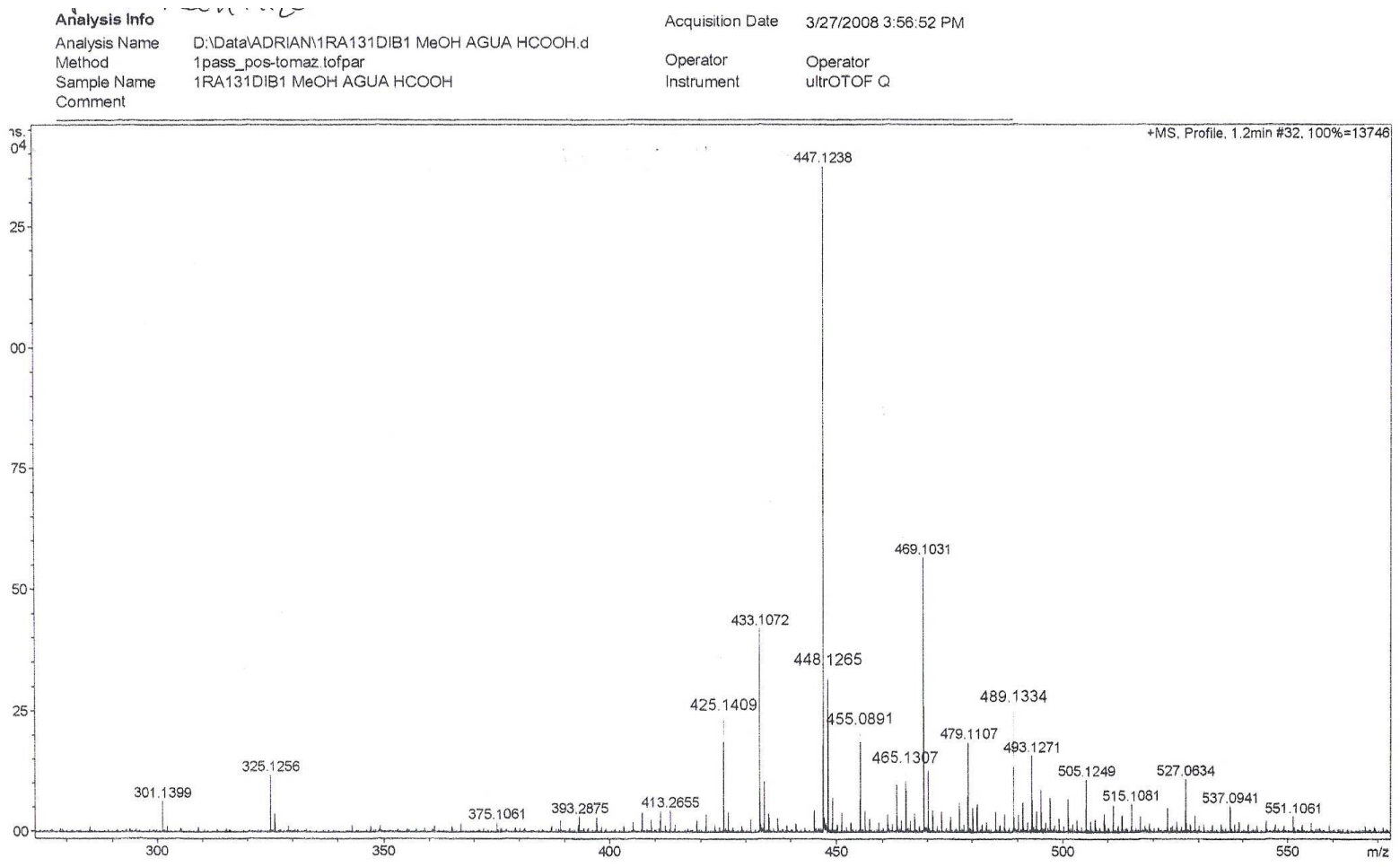


Figura 10. Espectro de massas (ESI-MS) do derivado semi-sintético **25**.

O derivado **26**, obtido por reação de hidrólise alcalina da neosergeolida (**5**) apresentou r.f. de 0,35 mm, tempo de retenção de 2 min e 5 seg., teor de pureza do pico maior do que 98%, com UVmáx de 245 nm. O espectro de massas obtido (ESI-MS, CH_3OH:H_2O:HCO_2H) apresentou fragmentações (m/z) em 449,1493 $[M + H]^+$, 471,1328 $[M + Na]^+$ e 489,1417 $[M + Na + H_2O]^+$ compatíveis com a estrutura proposta para o derivado **26**.

2.3.3 - Epoxidação

O derivado **27**, obtido por reação de epoxidação da isobruceína B (**2**), apresentou fator de retenção (r.f.) de 0,60 mm por CCD analítica e tempo de retenção de 5 min e 28 seg. por CLAE, teor de pureza do pico maior do que 98% e UVmáx de 245 nm. Já o derivado **28**, obtido por reação de epoxidação da neosergeolida (**5**) apresentou r.f. de 0,42 mm, tempo de retenção de 9 min e 52 seg., teor de pureza do pico maior do que 98% e UVmáx de 278 nm.

2.3.4 - Benzoilação

Os derivados **29** e **30**, obtidos por reação de benzoilação da isobruceína B (**2**), apresentaram r.f. de 0,69 e 0,71 mm por CCD analítica, respectivamente e tempo de retenção de 12 min e 53 seg e 11 min e 22 seg, respectivamente por CLAE, teores de pureza dos picos maiores do que 98% e UVmáx de 244 e 198 nm, respectivamente. Os derivados **31** e **32**, obtidos por reação de benzoilação da neosergeolida (**5**) apresentaram r.f. de 0,73 e 0,79 mm por CCD analítica, respectivamente e tempo de retenção de 13 min e 2 seg. e 11 min e 37 seg.., respectivamente por CLAE, teores de pureza dos picos maiores do

que 98% e UVmáx de 247 e 207 nm, respectivamente. Já os derivados **33** e **34**, obtidos por reação de benzoilação da 15-desacetil neosergeolida (**24**) apresentaram r.f. de 0,64 e 0,67 mm por CCD analítica, respectivamente e tempo de retenção de 13 min e 14 seg. e 11 min e 41 seg., respectivamente por CLAE, teores de pureza dos picos maiores do que 98% e UVmáx de 247 e 194 nm, respectivamente.

2.3.5 - Redução

O derivado **35**, obtido por reação de redução da isobruceína B (**2**), apresentou fator de retenção (r.f.) de 0,41 mm por CCD analítica e tempo de retenção de 5 min e 1 seg.. por CLAE, teor de pureza do pico maior do que 98% e UVmáx de 247 nm. O derivado **36**, obtido por reação de epoxidação da neosergeolida (**5**) segundo o mesmo protocolo acima, apresentou r.f. de 0,60 mm, tempo de retenção de 9 min e 52 seg. por CLAE, teor de pureza do pico maior do que 98% e UVmáx de 276 nm. O espectro de massas obtido (ESI-, MeOH:H_2O (80:20), Figura 12) apresentou fragmentação (m/z) em 449,3 $[M - H]^+$ compatível com uma das estruturas propostas para o derivado **36**, denominadas **36a** e **36b** (Figura 11).

2.4 - Ensaios de atividade biológica

2.4.1 - Ensaio de toxicidade frente a larvas de *A. franciscana*

O ensaio com larvas do microcrustáceo *A. franciscana* é um método simples e barato que permite avaliar a toxicidade geral e pode ser considerado um ensaio preliminar no estudo de extratos e substâncias puras para determinação da atividade antitumoral. O

derivado **25**, apesar de ter apresentado CI_{50} aproximadamente 7 vezes menor do que o quassinóide natural isobruceína B (**2**) (Tabela 2), ainda pode ser considerado bastante ativo o que o coloca como uma substância promissora para futuros testes de atividade citotóxica frente a células tumorais.

Tabela 2 - Atividade tóxica das frações obtidas por partição do extrato etanólico de frutos de *P. sprucei* frente a larvas de *A. franciscana*.

Amostra	CI_{50} (μg/mL)
2	1,3*
5	0,9*
25	8,8
1RA10	64,0
3RA16C	130,0
3RA16P	24,0
Fr. hexânica[1]	128,0
Fr. clorofórmica[1]	n.d.
Fr. ac. etila[1]	n.d.
Fr. n-butanol[1]	6,0
Fr. aquosa[1]	>130,0

1 - Frações obtidas a partir da partição do extrato etanólico dos frutos inteiros de *P. sprucei*.
n.d. – não determinado. *Silva, 2006.

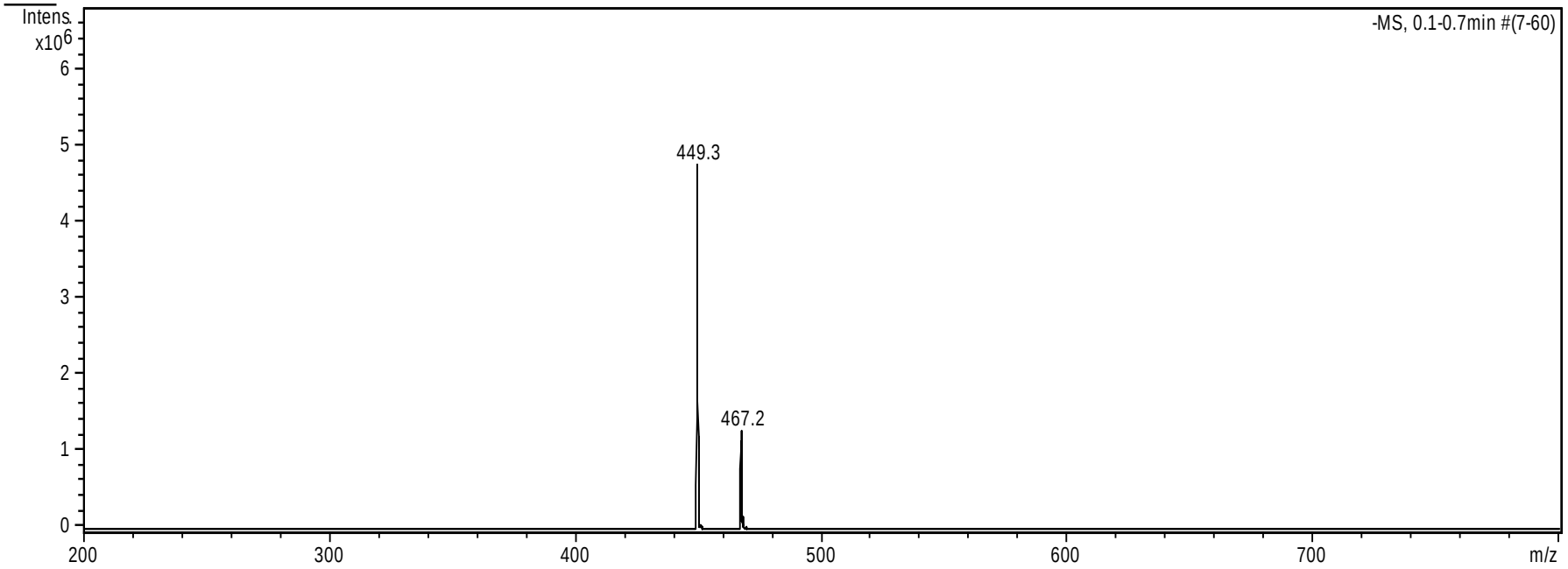


Figura 11. Espectro de massas (ESI-MS) do derivado semi-sintético **36**.

2.4.2.2 - Avaliação da atividade inseticida frente a imagos de *A. aegypti*

Devido à limitação de quantidade de amostra, até o presente momento somente os quassinóides naturais (**2**) e (**5**) foram submetidos a este teste. Apesar da significativa atividade larvicida, não se observou atividade inseticida frente às espécies estudadas, até uma concentração de 750 μg/mL. Entretanto, acreditamos que a dificuldade de ingestão das substâncias pelo inseto mascarou possíveis efeitos tóxicos. Novos testes devem ser realizados com o uso de um veículo que permita a entrega da droga teste aos insetos.

2.4.3 - Ensaio de citotoxicidade frente a células tumorais *in vitro*

Os extratos e frações obtidas a parti dos frutos inteiros bem como das cascas e polpas dos frutos de *P. sprucei* já foram testadas frente a quatro linhagens tumorais e estão em fase final de cálculo das CI_{50}. Como pode ser observado na Tabela 3, somente a fração hexânica obtida a partir da partição do extrato etanólico dos frutos inteiros de *P. sprucei* não apresentou boa atividade citotóxica. Todas as demais se mostraram bastante tóxicas a pelo menos uma das linhagens testadas.

A análise das CI_{50} das frações obtidas a partir dos extratos de cascas e polpas dos frutos de *P. sprucei* mostra que, tanto a fração clorofórmica das cascas quanto a fração clorofórmica da polpa foram bastante ativas, com valores de CI_{50} iguais ou inferiores a 0,92 μg/mL (Tabela 4). Entretanto, a fração n-butanólica da casca/polpa foi bastante ativa (CI_{50} 0,78-1,82 μg/mL) enquanto que a fração n-butanólica da casca/polpa foi inativa até uma concentração de 100 μg/mL, dando uma boa indicação de que a composição química

distinta entre essas frações. A forte atividade citotóxica apresentada por essas frações indica que o estudo fitoquímico das mesmas deve revelar substâncias de elevado potencial antitumoral.

Tabela 3 - Atividade citotóxica das frações obtidas por partição do extrato etanólico de frutos inteiros de *P. sprucei* frente a células tumorais *in vitro*.

Amostra	Linhagem		
	HCT-8	SF295	MDA-MB435
	Classificação da atividade (atividade citotóxica %)		
Fr. hexânica	PA (22,43%)	PA (29,77%)	PA (28,44%)
Fr. clorofórmica	MA (88,50%)	PA (17,64%)	MA (85,08%)
Fr. ac. etila	MA (84,91%)	MA (87,83%)	MA (85,60%)
Fr. n-butanólica	MA (82,39%)	MA (86,73%)	MA (84,98%)
Fr. aquosa	MA (83,79%)	PA (20,23%)	MO (69,03%)

Como critério de atividade *in vitro* foi estabelecido que as amostras que inibirem o crescimento dos parasitos de: 80 a 100% = muito ativa (MA); 50 a 79% = parcialmente ativa (PA); < 50% = pouco ativa (PA).

2.4.5 - Ensaio de inibição da atividade mitocondrial

Observou-se que os quassinóides isobruceína B (**2**) e neosergeolida (**5**) não foram capazes de alterar a capacidade de mitocôndrias extraídas de feijão de metabolizarem os

substratos succinato e malato até uma concentração de 60 µM/mL. Dessa forma, as referidas atividades biológicas testadas até o momento não possuem mecanismo de ação envolvendo alterações do metabolismo respiratório em células vegetais.

Tabela 4 - Atividade citotóxica das frações obtidas por partição dos extratos etanólicos de cascas e polpas de frutos de *P. sprucei* frente a células tumorais *in vitro*.

Amostra	Linhagem		
	HCT-8	SF295	HL-60
	CI_{50} (µg/mL)		
Polpa			
Fr. hexânica	n.d.	n.d.	n.d.
Fr. clorofórmica	<0,19	<0,19	<0,19
Fr. ac. etila	n.d.	n.d.	n.d.
Fr. n-butanólica	1,37	1,82	0,78
Fr. aquosa	31,87	91,35	27,43
Cascas			
Fr. hexânica	n.d.	n.d.	n.d.
Fr. clorofórmica	0,11	0,92	0,30
Fr. ac. etila	n.d.	n.d.	n.d.
Fr. n-butanólica	>100	>100	>100
Fr. aquosa	n.d.	n.d.	n.d.

n.d. – não determinado

2.4.6 - Ensaio de atividade hemolítica

Da mesma forma que para o ensaio de inibição da atividade mitocondrial, nenhuma das amostras testadas (quassinóides naturais e extratos e frações do fruto, caule e raiz de *P. sprucei*) não apresentaram atividade hemolítica até uma concentração de 500 µg/mL, indicando que não há envolvimento entre as atividades biológicas apresentadas e o rompimento de membranas biológicas.

2.4.7 - Ensaio de atividade antimalárica

Uma significativa inibição do crescimento do parasita causador da malária humana *P. falciparum in vitro* foi exibida pelos quassinóides (**1**) e (**2**) (Tabela 5)**.** A neosergeolida (**2**) e a isobruceína B (**1**), exibiram as maiores atividades, aproximadamente 15 a 82 vezes maiores do que as apresentadas pela quinina e cloroquina e similares à apresentada pela artemisinina. Se a mesma comparação for analisada numa escala molar, os quassinóides (**1**) e (**2**) apresentaram atividades de 15 a 445 vezes maior do que a quinina e a cloroquina. A sensibilidade *in vitro* do *P. falciparum* às substâncias testadas foi similar e reprodutível em ensaios realizados em duplicatas em ocasiões separadas. Alguns requerimentos estruturais tais como a presença de uma cetona α,β-insaturada no anel A, uma ponte epoximetileno no anel C e a presença de uma função éster em C-15 são considerados muito importantes para a atividade antimalárica apresentada pelos quassinóides (Okano et al. 1995) e estão presentes nos dois quassinóides testados no presente trabalho. Desta forma, estamos

demonstrando também que os quassinóides (**2**) e (**5**) são, pelo menos em parte, responsáveis por esta atividade biológica apresentada pelos chás de *P. sprucei*.

Os estudos preliminares não indicam efeito sinérgico entre a isobruceína B (**2**) e a neosergeolida (**5**). Entretanto, ainda está sendo estudado o possível efeito sinérgico entre os quassinóides e os antilamaláricos comerciais quinina, cloroquina e artemisinina.

Da mesma forma que foi observada nos ensaios de atividade citotóxica, os ensaios de atividade antimalárica das frações do fruto de *P. sprucei* (Tabela 6) indicam um forte potencial antimalárico dos constituintes que por ventura venham a ser isolados e caracterizados.

2.4.9 - Ensaios de atividade antibacteriana, antifúngica e anti-helmíntica

Os quassinóides (**2**) e (**5**) apresentaram fraca atividade antimicrobiana, tanto frente às espécies bacterianas *A. hydrophila, B. subtilis, P. aeruginosa* e *S. aureus*, com halo de inibição vadiando de 6,5 a 8,0 mm, cerca de quatro a cinco vezes menor do que a norfloxacina (controle positivo), quanto frente à espécie fúngica *A. ochraceus* com concentração inibitória mínima superior à dose máxima testada de 570 ppm (Tabela 7), indicando, portanto, um fraco potencial antimicrobiano dos referidos quassinóides. Da mesma forma, os respectivos quassinóides apresentaram apenas fraca atividade antihelmíntica frente ao nematóide *M. exigua*, indicando uma relativa seletividade, visto que já havia sido reportada uma significativa atividade anti-helmíntica dos mesmos quassinóides frente ao nematóide *M. contortus*, um nematóide parasítico do aparelho gastrointestinal de ruminantes domésticos e silvestres com inibição *in vitro* de 72 e 77%, respectivamente a uma concentração de 86 ppm.

Tabela 7 - Atividade antimicrobiana e anti-helmíntica dos quassinóides naturais obtidos de *P. sprucei*.

Substância	Atividade Biológica					
	Anti-helmíntica[a]	Antibacteriana[b]				Antifúngica[c]
	M. exigua	*A. hydrophila*	*B. subtilis*	*P. aeruginosa*	*S. aureus*	*A. ochraceus*
2	26,2	6,5	7,5	-	7,0	> 570
5	25,2	8	7,5	7	-	> 570
Tween 80	2,2	n.d.	n.d.	n.d.	n.d.	n.d.
Norfloxacina	n.d.	32	31	26	24	n.d.
Cloreto de Benzalcônio	n.d.	n.d.	n.d.	n.d.	n.d.	7,7

a – Percentagem de juvenis mortos a uma concentração de 500 ppm; b - Halo (mm), a uma concentração de 500 ppm; c – Concentração inibitória mínima (CIM) (ppm); n.d. = não determinada.

Conclusões

- ✓ Um novo e seletivo método para obtenção do quassinóide neosergeolida (**5**) foi obtido. Entretanto, ainda há a necessidade de aperfeiçoamento de tal procedimento a fim de se obter seqüencialmente elevado teor do quassinóide isobruceína B (**2**), mantendo a simplicidade, rapidez, baixo custo e baixo requerimento de solventes muito tóxicos tais como os solventes clorados.
- ✓ Os quassinóides **1** e **2** apresentaram elevada atividade antimalárica, ao passo que seus derivados acetilados apresentaram se bem menos ativos.
- ✓ Estudos de mecanismo de ação demonstraram que **2** apresenta forte atividade citotóxica devido à indução da apoptose das células tumorais.
- ✓ Os quassinóides **1** e **2** apresentaram fraca atividade antimicrobiana e anti-helmíntica e moderada atividade larvicida frente a larvas de *A. aegypti*.
- ✓ Infusões do caule de *P. sprucei* apresentaram significativo teor de quassinóides.
- ✓ Foram preparados 14 derivados semi-sintéticos, sendo 3 já conhecidos e 11 inéditos, dentre os quais somente os derivados acetilados já tiveram sua elucidação estrutural completa. Todos os demais estão em fase de análise.
- ✓ Apesar de se encontrar em fase inicial, o estudo fitoquímico dos frutos de *P. sprucei* já resultou no isolamento de uma substância (em fase de elucidação estrutural) e na apresentação de uma gama de atividades biológicas inéditas para o fruto. Apenas as frações clorofórmicas obtidas da semente e da estrutura protetora da semente (epicarpo, tegumento e mesocarpo) dos frutos de *P. sprucei* apresentaram teor de neosergeolida e isobruceina B quantificável pela técnica de CCD-densitometria e este teor é

relacionado às atividades biológicas estudadas. Entretanto a presença de outras substâncias também revelou um elevado potencial citotóxico para as demais frações.

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