UNIVERSIDADE ESTADUAL PAULISTA "JÜLIO DE MESQUITA FILHO"
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AVALIAÇÃO DA SEGURANÇA E DA ATIVIDADE IMUNOMODULADORA DOS EXTRATOS DE *UNCARIA TOMENTOSA* E *UNCARIA GUIANENSIS* SOBRE A PATOGENIA DO DIABETES MELITO AUTOIMUNE INDUZIDO PELA ESTREPTOZOTOCINA.

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Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP, como parte dos requisitos para obtenção do título de Doutor.

BOTUCATU, SP

2011

Dedico esses quatro anos de trabalho aos meus pais Adalberto e Dalva. Trinta anos de suporte e muita batalha, sem os quais eu não teria chegado a esse momento. À minha irmã Paula e meus avós José, Maria, Lázaro e Jandira. O reflexo dos seus valores e da sua dedicação encontram-se em cada página dessa tese.

Dedico ainda cada página desse trabalho a minha namorada Daniela. Conviver com alguém tão plena em seus atributos é o maior presente do mundo. Sem você nada disso seria possível. Te amo !

Agradecimentos

À Dra. Rosa Marlene Viero e a Dra. Alexandrina Sartori, minhas orientadoras e grandes amigas, por terem acreditado e assumido o desafio da execução do projeto. Sem o seu voto de confiança nada disso teria sido realizado. Em todas as conquistas que eu venha a obter, serei sempre grato a vocês.

Ao Dr. João Lauro Viana de Camargo, pelo apoio, incentivo e lições de profissionalismo, uma constante inspiração. Por possibilitar o uso do laboratório Toxicam, seu parque de equipamentos e serviços técnicos.

Aos Drs. Antonio Carlos Siani e Lígia M. M. Valente, por toda a ajuda referente à obtenção dos extratos e discussão dos artigos.

À Marjorie Golim, pela ajuda nas análises de citometria de fluxo.

Ao amigo Paulo César Georgete (PC), por cuidar dos nossos animais e todo o auxílio durante o período no biotério.

A Paulo e Mara, do laboratório Toxicam. Agradeço o seu trabalho árduo e sua amizade, a qual sempre me ajudou em momentos difíceis.

Aos colegos de pós-graduação, pela convivência nesses anos todos.

Aos meus grandes amigos Milton, Douglas, Alysson, Bill, Rafael, Spike. Poucas pessoas podem manter amizades que inciaram-se há 30 anos.

À Bali, minha companheira canina que me ensina constantemente o significado da doação.

À Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, pelo apoio ao projeto por meio do auxílio à pesquisa.

À Coordenação e Aperfeiçoamento de Pessoa de Nível Superior, CAPES, pelo apoio financeiro através da bolsa de doutorado.

À Coordenadora do Curso de Pós-Graduação em Patologia, Dra. Márcia Guimarães. Pela amizade e apoio em momentos cruciais.

Aos funcionários do Departamento de Patologia, pela convivência e apoio durante esse período.

Finalmente, a todos que de alguma forma contribuíram durante o desenvolvimento do meu mestrado.

"Imagination is more important than knowledge. Knowledge is limited.

Imagination encircles the world." – Albert Einstein

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INTRODUÇÃO

1. Introdução

1.1. A natureza como fonte de novas estratégias terapêuticas

Desde o seu alvorecer, a humanidade utiliza-se da natureza com a finalidade de satisfazer uma gama de necessidades, abrangendo desde a produção de alimentos, vestuário, meios de transporte e fragrâncias até a cura e/ou prevenção de doenças (Gurib-Fankin, 2006). Os primeiros documentos relatando o uso de plantas com propósitos terapêuticos datam de cerca de 78 anos antes de Cristo (A.C), quando Pedáneo Dioscórides, autor greco-romano fundador da farmacognosia, escreveu a obra " De Matéria Medica", descrevendo milhares de plantas medicinais cuja utilização ainda é observada nos tempos modernos (López-Muñoz et al., 2006).

Durante séculos, as propriedades medicinais de diversas espécies vegetais foram confirmadas empiricamente e passadas de geração a geração, tornando-se a base da medicina tradicional em diversos países como o Brasil (Johann et al., 2010), Índia (Ahmed e Urooj, 2010) e China (Xiang et al., 2010). Diversas espécies vegetais utilizadas tradicionalmente apresentam atividades farmacológicas marcantes, incluindo efeitos antitússico (*Althaea officinalis)* (Basch et al., 2003), cicatrizante (*Calendula officinalis)* (Basch et al., 2006), antidepressivo *(Centella asiática)* (Brinkhaus et al., 2000), antiviral (*Echinacea purpúrea)* (Tierra, 2007), ansiolítico (*Passiflora incarnata)* e imunomodulador (*Uncaria tomentosa) (Valerio e Gonzales, 2005).* Apesar das raízes empíricas, muitas plantas medicinais tornaram-se fontes de novas classes terapêuticas. Relatos da medicina egípcia apontam o uso da espécie *Ammi majus* (Cicuta negra) para o tratamento do vitiligo. Recentemente, a droga β-methoxypsoralen foi desenvolvida a partir dessa espécie, sendo utilizada para o tratamento de desordens cutâneas e do linfoma de células T (Bartosová et al., 2006). De forma semelhante, a observação de

que frações alcalóides da espécie *Catharanthus rósea* (Vinca) causam leucopenia severa em ratos levou ao desenvolvimento dos agentes quimioterápicos Vincristina e Vinblastina. Atualmente, essas drogas representam terapias importantes para o tratamento da leucemia aguda, linfoma de Hodgkin´s e tumor testicular metastático (Levêque e Jehl, 2007).

Atualmente, aproximadamente metade das espécies vegetais do planeta é encontrada em florestas tropicais, reconhecidas como verdadeiras bibliotecas de moléculas com potencial terapêutico (Cravotto et al., 2010). A possibilidade de descoberta de novas drogas a partir de espécies nativas dessas regiões é enorme, visto que apenas cerca de 1% das espécies tropicais foram adequadamente caracterizadas com relação ao seu potencial farmacológico (Gurib-Fakim, 2006). Além disso, a existência de plantas nativas ainda desconhecidas pela ciência ressalta a importância da preservação das regiões de florestas tropicais. Conjuntamente, os países da América Latina congregam uma grande parte da biodiversidade mundial. Nesse cenário, o Brasil apresenta 20 a 22% de todas as plantas e microorganismos (Calixto, 2000).

O uso de plantas medicinas ainda é um fator preponderante em muitos sistemas de saúde ao redor do mundo, particularmente em países em desenvolvimento como Brasil, Peru, Índia e China (Balbani et al., 2009; Bussmann et al., 2010; Kumar et al., 2007). Estima-se que cerca de 80% da população africana e 40% da população chinesa amparam-se regularmente na medicina tradicional para cuidados básicos de saúde (WHO, 2002). Em paralelo, o impacto econômico da medicina alternativa é sentido cada vez mais em países desenvolvidos com um sistema de saúde bem estruturado, como Canadá e Reino Unido, apresentando lucros na ordem de 2,3 a 2,4 bilhões de dólares anuais, respectivamente (WHO, 2002). Estudos corroboram esses dados, revelando um aumento exponencial no uso de produtos vegetais a partir da década de 70

(Block and Mead, 2003). A crescente atenção dada aos produtos naturais por toda uma geração explica-se parcialmente pela redução da confiança sobre os medicamentos convencionais sintéticos, caracterizados por uma atividade rápida e potente, mas seguida em alguns casos por efeitos adversos importantes. Essa desconfiança sobre a segurança das drogas convencionais é ainda abastecida pela autoconfiança da população e a prática de auto-medicação, cujas conseqüências não raramente são deletérias à saúde humana (Gilson e Kreis, 2009).

É importante ressaltar que a medicina tradicional é muitas vezes a única opção de saúde disponível, especialmente para as populações mais pobres. Em Uganda, por exemplo, a proporção entre os praticantes da medicina tradicional e a população fica em torno de 1:200 a 1:400. Contudo, quando falamos sobre a proporção de médicos alopatas por habitante, os valores checam a 1: 20.0000, revelando a fragilidade do sistema de saúde e o papel fundamental das ervas medicinais no dia a dia das pessoas (WHO, 2002).

Diversas iniciativas foram iniciadas ao redor do mundo com o objetivo de reduzir taxas de mortalidade e morbidade através do estimulo ao uso de produtos vegetais. Como resultado, a Organização Mundial de Saúde (OMS) lançou o "Traditional Medicine Strategy 2002-2005" cujo objetivo é reparar a realidade de milhões de pessoas ao redor do globo para as quais os medicamentos não estão disponíveis ou apresentam custos exorbitantes. Assim, o objetivo dessa política é facilitar a integração da medicina tradicional, incluindo produtos vegetais, nos sistemas nacionais de saúde, produzindo guias relativos aos métodos de produção e sua padronização e estimulando pesquisas clinicas sobre sua eficácia e segurança, com ênfase para doenças epidêmicas e negligenciadas.

1.2. Plantas medicinais no âmbito da pesquisa farmacêutica

Após a Segunda Guerra Mundial, a pesquisa farmacêutica expandiu-se de forma exponencial, promovendo uma revolução na medicina moderna. Durante a segunda metade do século 20, a pesquisa de novas moléculas pela indústria farmacêutica apresentou momentos com o predomínio da síntese química e outros direcionados à prospecção de novos compostos a partir de produtos naturais (Potterat e Hamburger, 2008). Até o início da década de 90, estima-se que cerca de 80% das drogas disponíveis no mercado eram considerados produtos naturais ou estruturas análogas, abrangendo diversas classes terapêuticas, tais como agentes antibióticos (ex. penicilina, eritromicina, tetraciclina), imunosupressores (ciclosporina, rampamicina), antineoplásicos (taxol, doxorubicina) e cardiovasculares (captopril, digoxina) (Cragg et al., 1997). Contudo, com a otimização da síntese química, a participação de derivados naturais no mercado farmacêutico caiu para aproximadamente 25 a 30% durante o ano 2005 (Harvey, 2005). Apesar disso, treze medicamentos derivados de produtos naturais foram aprovados nos Estados Unidos durante o período referido, com cinco deles representando os primeiros membros de novas classes terapêuticas (Harvey, 2005).

Tendo em vista a importância dos produtos naturais para o desenvolvimento de novos medicamentos e o predomínio do seu uso pelas populações mais pobres, o que dificulta a adoção de extratos vegetais pelos sistemas públicos de saúde? Sem dúvida, esse é um assunto de grande complexidade, englobando fatores políticos, econômicos e sociais. Contudo, três pontos chamam a atenção. Em primeiro lugar, muitos países não apresentam políticas ou medidas regulatórias próprias relativas ao uso de plantas medicinais para os cuidados básicos de saúde. Além disso, para muitas espécies utilizadas tradicionalmente não existem estudos científicos adequados sobre sua

eficácia, segurança e qualidade. Outro ponto a ser observado é a falta de formação de médicos especialistas em medicina tradicional ou a atualização de médicos alopatas sobre o emprego dessas substâncias na rotina. (WHO, 2002).

Outras dificuldades de caráter estratégico e econômico dificultam o desenvolvimento de novos medicamentos a partir de produtos naturais. O "screening" de novas moléculas a partir de fontes naturais pela indústria farmacêutica vem encontrando algumas limitações importantes, particularmente durante a última década. A conjuntura econômica do setor cria a necessidade do desenvolvimento de drogas "blockbusters", com vendas superiores à cifra de 1 bilhão de dólares ao ano. Contudo, a expiração da patente de tais produtos representa uma queda de aproximadamente 80% nos lucros (Panattoni, 2010). Essa situação vem pressionando as grandes empresas farmacêuticas a desenvolverem novos medicamentos de forma precisa, rápida e com o menor gasto em pesquisa e desenvolvimento (P&D) possível. Nesse contexto, produtos naturais não são considerados como os melhores candidatos para o "high-throughput screening" (HTS) de drogas com atividade terapêutica adequada (Koehn e Carter, 2005). Essa situação pode ser explicada pelo fato de que, ao contrário da biblioteca sintética, moléculas derivadas de produtos naturais normalmente apresentam estruturas complexas, caracterizadas pela presença de diversos substituintes contendo oxigênio e diversos centros de isomeria óptica (Butler, 2004). Essas características normalmente criam entraves e desaceleram o processo de identificação de novos candidatos com atividade farmacológica otimizada. Outras dificuldades no HTS a partir de produtos naturais incluem a qualidade da matéria prima, o impacto de fatores ambientais e climáticos durante o cultivo das espécies e a disponibilidade de informações tradicionais e/ou científicas sobre sua eficácia e segurança (Ernst, 2005; Boullata e Nace, 2000). É amplamente reconhecido que um dos maiores impedimentos enfrentados ao longo dos

anos na adoção de extratos vegetais pela medicina moderna é a grande variabilidade da qualidade dos produtos (Vlietinck et al., 2009). Em contrate com as substâncias sintéticas, materiais botânicos estão constantemente sujeitos a variações do conteúdo fitoquímico. Fatores como a característica genética da espécie, condições do solo e clima e incidência de pestes influenciam diretamente na qualidade do produto final (Calixto, 2000). Além disso, as condições de colheita, secagem e armazenamento do material podem afetar de maneira importante a qualidade final do produto vegetal (Vlietinck et al., 2009). Um estudo realizado com diversas amostras comerciais de *Panax ginseng* e *Panax quinquefolius*, disponíveis no mercado norte-americano durante os anos 1995 a 1998, revelou uma variação dos marcadores químicos de 0.00% a 13.54% e 0.009% a 8.0%, respectivamente. Além disso, 26% das amostras sequer obedeciam às especificações disponíveis nos respectivos rótulos (Li et al., 2008). Durante o processo de "modernização" no uso de plantas medicinais, a reprodutibilidade da qualidade das matérias primas é alvo de constante debate. Tradicionalmente, a identificação de produtos vegetais é baseada segundo sua morfologia, presença de um ou dois marcadores e/ou determinação do seu conteúdo químico. Contudo, essa metodologia pode não fornecer um perfil adequado da droga, dificultando a distinção de produtos com aparência similar e/ou conteúdo químico semelhante (Jiang et al., 2010). A característica de múltiplos alvos farmacológicos e o sinergismo observado através do uso de extratos vegetais está diretamente relacionada com o seu rico conteúdo fitoquímico. Assim, metodologias que abordem a variabilidade fitoquímica desses extratos devem ser adequadamente conduzidas, especialmente se essas variações possuem correlação com sua segurança e eficácia farmacológica.

1.3. Fatores que impactam a segurança de fitoterápicos

1.3.1. Aspectos regulatórios

O estudo de plantas medicinais vem recebendo uma atenção crescente no Brasil, não apenas pela comunidade acadêmica, mas também por grandes indústrias farmacêuticas e agências regulatórias. O objetivo central desse movimento é o desenvolvimento de compostos com eficácia, segurança e qualidade comprovada.

Segundo a RDC número 14/10 da Agência Nacional de Vigilância Sanitária (ANVISA), fitoterápicos são medicamentos obtidos utilizando-se exclusivamente matérias-primas ativas vegetais, cuja eficácia e segurança são validadas por meio de levantamentos etnofarmacológicos, dados científicos ou evidências clínicas. Não podem ser incluídas na formulação de medicamento fitoterápico substâncias ativas isoladas, de qualquer origem, nem as associações destas com extratos vegetais (ANVISA, 2010). Durante o processo de registro, a comprovação da eficácia e segurança de um fitoterápico pode ser realizada de quatro maneiras: 1) obtenção de pontuação definida segundo a apresentação de estudos farmacológicos e toxicológicos presentes nas monografias contidas na "Lista de Referências bibliográficas para avaliação de segurança e eficácia de fitoterápicos". 2) apresentação segundo o guia para ensaios toxicológicos pré-clínicos, que estabelece os critérios mínimos aceitáveis para os estudos toxicológicos agudo, sub-crônico e crônico, testes para medicamentos de uso tópico e o estudo especial de genotoxicidade. Para os estudos clínicos, devem ser seguidas as determinações do Conselho Nacional de Saúde (CNS). 3) apresentação de levantamento bibliográfico etnofarmacológico, mostrando a eficácia e a segurança do produto que tenha uso comprovado por um período igual ou superior a 20 anos. 4) lista de medicamentos fitoterápicos de registro simplificado que contempla 36 espécies vegetais

para as quais é dispensada a comprovação da eficácia e segurança, considerando a quantidade de estudos que já foram publicados sobre cada uma dessas espécies (ANVISA, 2010).

Medicamentos fitoterápicos, assim como drogas convencionais, possuem uma série de princípios ativos que podem induzir efeitos-adversos significativos quando ingeridos em concentrações inadequadas. Como dito anteriormente, na prática, produtos vegetais apresentam uma grande variabilidade de constituintes químicos, tornando necessário o estabelecimento de faixas de concentração mínima e máxima para assegurar o efeito terapêutico esperado e a fim de se evitar a incidência de toxicidade (Chavez e Jordan, 2006). O processo de padronização de extratos vegetais é um passo extremamente importante para assegurar a presença e concentrações adequadas de grupos químicos característicos de uma dada espécie, possibilitando assim a confecção de dossiês confiáveis relativos à sua eficácia e/ou segurança (Van Breemen et al., 2008).

Ao longo dos anos, diversos estudos têm demonstrado a toxicidade de produtos botânicos. China e Taiwan promoveram diversas restrições ao uso de diversas espécies, incluindo *Typhonium giganteium* e *Pinellia ternata* (tubérculos), *Garcinia morella* (resinas), *Impatiens balsamina*, *Pharbitis nil* e *Picea purpúrea* (sementes), *Knoxia valerianoides* (raízes) e *Rhododendron molle* (flores) (De Smet, 2004). Existem diversos modos pelos quais produtos vegetais podem causar toxicidade, incluindo um efeito tóxico direto, interações entre plantas e drogas convencionais e a presença de adulterantes em sua fórmula (De Smet, 2004). Ainda hoje, os dados disponíveis na literatura científica relativos à toxicidade de produtos naturais são escassos ou insuficientes. Williamson (2003) propôs uma classificação do potencial tóxico de produtos vegetais, com ênfase para as interações entre plantas e drogas convencionais: 1) Toxicidade confirmada: diversos estudos com lotes distintos da espécie

demonstrando um efeito tóxico, 2) Potencialmente tóxico: 1 ou mais estudos experimentais e clínicos demonstrando algum tipo de toxicidade, 3) Teoricamente tóxico: evidências teóricas sem a confirmação clínica, 4) Seguro: ausência de toxicidade em condições racionais de uso, 5) Reporte de caso: dados isolados sobre a toxicidade ou interação entre plantas e drogas, sugerindo a necessidade de estudos adicionais.

Para se determinar de maneira adequada a toxicidade de um produto vegetal, pesquisadores e médicos deveriam trabalhar conjuntamente a fim de esclarecer se o efeito observado pode ou não estar relacionado ao uso de uma planta medicinal. Alguns pontos a serem observados incluem o histórico médico do paciente, informações sobre o local no qual o produto foi obtido (mercados populares, laboratórios especializados), a forma, dose e frequência de uso (chás, comprimidos, cápsulas) (Tovar, 2009). Uma vez identificado o produto consumido, outros fatores devem ser levados em conta, incluindo a presença de contaminantes (metais pesados, drogas sintéticas), processamento e preparo inadequado, interações droga-planta, doenças coexistentes e identificação incorreta da espécie (Ko, 2006).

1.3.2. A presença de contaminantes nas formulações vegetais influenciando a incidência de eventos adversos

Relatos sobre a presença de adulterantes e/ou contaminantes em formulações vegetais infelizmente são muito comuns na literatura. Huang et al.. (2008) apontou uma taxa de adulteração em 23.7% das 2609 espécies de plantas chinesas avaliadas. Nesses casos, os tipos de adulterantes mais observados incluíram a cafeína, indometacina, acetaminofeno, hidroclorotiazida, predinisona e colchicina. Uma dica comumente utilizada durante a pesquisa de adulterantes em produtos naturais é verificar o tipo de

atividade terapêutica aventada pelo fabricante. Por exemplo, no caso de um composto indicado para o controle do diabetes, deve-se procurar por medicamentos orais hipoglicemiantes (sufoniluréias e biguanidas). É importante observar que, não raramente, os efeitos tóxicos observados após a ingestão de um produto vegetal podem estar diretamente relacionados à presença desses compostos adulterantes, particularmente quando ocorre uma reação cruzada, sinérgica ou antagônica, entre possíveis medicamentos alopáticos que o paciente faz uso e os adulterantes presentes em uma fórmula vegetal (Sucher e Carles, 2008). Outro ponto sensível reside no fato de que cerca de 50% dos pacientes não informam aos seus médicos o uso de plantas medicinais durante o tratamento alopático (Sucher e Carles, 2008). Essa falha de comunicação dificulta os médicos na identificação dos eventos adversos relativos a um produto vegetal ou possíveis contaminantes presentes na fórmula.

Uma revisão da literatura realizada por Koh e Woo (2000) avaliando diversas plantas medicinais chinesas durante os anos 1990 a 1997, relatou a presença de metais pesados como chumbo, mercúrio, cádmio, arsênico e tálio. Outros estudos demonstraram concentrações excessivas de potássio, sódio, níquel, magnésio, manganês, ferro, cobre, zinco, cromo e molibdênio (Koh e Woo, 2000). A farmacopéia européia procurou limitar o nível de contaminação de plantas medicinais por metais pesados, propondo um limite de 5 mg kg^{-1} para o chumbo, 0.5 mg kg^{-1} para o cádmio e 0.1 mg kg^{-1} para o mercúrio. A limitação da concentração de metais pesados em produtos vegetais também pode ser calculada através do "Nível de Ingestão Semanal Tolerável", estabelecido pela WHO. Nesse caso, a ingestão máxima para o mercúrio é de 5 μg kg^{-1} , para o arsênico é de 15 μg kg^{-1} , para o chumbo é de 25 μg kg^{-1} e para o cádmio é de 7 μg kg^{-1} (WHO, 1978; WHO 1989; WHO, 2000). Atenção particular deve ser dada para o chumbo e o mercúrio, devido sua a capacidade em se difundir através da

placenta, causando fetotoxicidade (Caldas e Machado, 2004). É importante salientar que apesar de todos os metais serem potencialmente tóxicos segundo os níveis de exposição, alguns são essenciais para funções fisiológicas e manutenção da homeostasia do organismo. Por exemplo, o zinco é um co-fator para mais de 100 metaloenzimas, sendo essencial para o desenvolvimento, reprodução e sistema imunológico (Kosalec et al., 2009). A contaminação de plantas medicinais por metais pesados pode ocorrer em qualquer fase de sua produção, incluindo emissões de fábricas nas regiões de cultivo e uso de praguicidas (Lynch e Braithwaite, 2005). É interessante notar que a medicina tradicional Chinesa e Indiana acredita nas propriedades terapêuticas de alguns metais pesados, adicionando níveis elevados desses compostos em muitas formulações vegetais (Lynch e Braithwaite, 2005). A despeito de uma contaminação proposital ou secundária por metais pesados, essa realidade também pode ser observada em países como o Brasil. Um estudo conduzido por Caldas e Machado (2001) revelou elevada ingestão de chumbo através da castanha-da-índia superior a 440% do "Nível de Ingestão Semanal Tolerável", o que poderia resultar em um acúmulo excessivo do metal no organismo caso o produto seja consumido durante um logo prazo.

Outra importante fonte de contaminação de produtos vegetais são os pesticidas. Abou-Arab e Abou Donia (2000) revelaram o predomínio do malathion em ervas medicinais, com os maiores níveis encontrados na camomila (2.19 mg kg^{-1}). No Brasil, amostras do gênero *Passiflora* (maracujá) revelaram contaminação por pesticidas organoclorados (dieldrin, lindane, tetradifon, chlorothalonil e endosulfan) em níveis que variam entre 21 e 71.4 μg kg^{-1} (da Silva et al., 2007). A presença de resíduos de pesticidas em produtos naturais industrializados é impactado diretamente pela relação entre a solubilidade do contaminante com os veículos de extração. Diversas metologias destinadas à avaliação da contaminação de produtos vegetais por pesticidas estão

descritas, incluindo a cromatografia líquida de alta performance e espectrometria de massa (WHO, 2004). Contudo, essas técnicas não são universalmente aplicadas e há a necessidade da execução métodos de separação pelos quais os resíduos de pesticidas podem ser concentrados e avaliados. Uma vez que o processo de separação seja incompleto, degrade o composto desejado ou promova a presença de metabólitos, a identificação dos contaminantes pode ser impactada de forma negativa.

Outra forma comum de contaminação observada em extratos vegetais é a presença de material biológico (bactérias, fungos, vírus, parasitas) ou produtos do metabolismo microbiano. A principal fonte de contaminação biológica das amostras vegetais decorre do ambiente de cultivo ou do processamento da matéria-prima (contaminação do solo, água, ar, humana) (WHO, 2004). Dados provenientes das farmacopéias européias e americanas especificam de forma bastante clara as regras para o controle de qualidade relativa aos contaminantes biológicos em produtos naturais (Vlietinck et al., 2009). A contagem de microorganismos aeróbicos e fungos (apresentados como unidades formadores de colônia por grama ou mililitro), ausência de salmonela, *Escherichia coli* e bactérias gram negativas tem sido utilizada como um indicador de controle microbiológico (Kosalec et al., 2009). O controle microbiológico dos produtos vegetais deve ainda levar em conta os seguintes aspectos: rotas de aplicação, presença de substratos que podem facilitar a proliferação de microorganismos, público alvo (recém nascidos, crianças, idosos), uso de agentes imunossupressores e presença de doenças secundárias debilitantes (Kosalec et al., 2009). A contaminação de amostras vegetais por microorganismos resistentes a antibióticos apresenta um risco particularmente grave. Um estudo conduzido por Brown e Jiang (2008) que avaliou 29 suplementos naturais obtidos nos Estados Unidos isolou as seguintes espécies resistentes à ampicilina, trimetropim, ácido nalidíxico ceftriaxona

e estreptomicina: *Bacillus spp*., *Erwinia spp*., *Ewingella americana*, *Staphylococcus spp*., *Enterobacter cloacae* e *Stenotrophomonas maltophilia*. Um estudo conduzido no Brasil com 91 amostras de plantas medicinas revelou a contaminação de 50% das amostras das partes aéreas e 16% das flores por algum tipo de fungo, com o predomínio dos gêneros *Aspergillus* e *Penicillium* (Bugno et al., 2006). Tendo em vista a toxicidade de metabólitos secundários produzidos por fungos, a legislação européia impôs limites de exposição para micotoxinas (aflatoxina B1, B2,G1 e G2 e ocratoxina) em diversos produtos naturais (Bugno et al., 2006).

1.3.3. Avaliação do potencial imunotóxico de plantas medicinais

A caracterização toxicológica de fármacos e produtos químicos diversos é um processo extremamente dinâmico e alvo de constante debate. Medidas regulatórias de âmbito nacional e internacional são constantemente publicadas e atualizadas a fim de se garantir a segurança de produtos industriais ou agentes ambientais sobre a saúde humana (Bass et al., 2009). Historicamente, os principais "endpoints" utilizados durante a avaliação toxicológica de produtos industriais estiveram relacionadas ao seu potencial cancerígeno e mutagênico (Snyder e Green, 2001; Brambilla et al., 2010). Contudo, com o passar dos anos e o desenvolvimento de novas metodologias, uma atenção crescente passou a ser voltada ao efeito de substâncias sobre o sistema imunológico (Descotes, 2006). De maneira geral, os efeitos imunotóxicos podem ser divididos em quatro categorias principais, denominadas imunossupressão, imunoestimulação, hipersensibilidade e auto-imunidade (Descotes, 2006).

Durante os últimos 25 anos, a imunotoxicologia tornou-se um braço extremamente importante da toxicologia e modelos experimentais *in vivo* e *in vitro*

permitiram a avaliação do potencial imunotóxico de diversos medicamentos (Luster e Gerberick, 2010). Para se estabelecer uma base científica para o uso de métodos de avaliação da imunotoxicidade, agências regulatórias, universidades e indústrias atuaram de forma conjunta na validação de estudos envolvendo camundongos e ratos (Schulte e Ruehl-Fehler, 2006). Atualmente, os testes preditivos em imunotoxicologia são realizados no contexto da toxicidade geral, de acordo com o guideline 407 da Organização para a Cooperação e o Desenvolvimento Econômico (OECD, 1995). A avaliação do sistema imunológico envolve aspectos hematológicos, incluindo a contagem diferencial de células, e a histopatologia de órgãos linfohematopoiéticos. Contudo, apesar desses parâmetros possibilitarem uma análise inicial da imunotoxicidade, eles não possibilitam avaliar a extensão dos efeitos observados sobre "endpoints" mais específicos do sistema imunológico, como subpopulações de linfócitos T e B, produção de anticorpos, proliferação celular, atividade de células "natural-killer" (NK) e testes de resistência do hospedeiro contra infecções e tumores (Luster e Gerberick, 2010). Os "guidelines" da European Medicines Agency (EMEA), Food and Drug Administration (FDA) e Ministry of Health, Labour and Welfare (MHLW) seguem uma política semelhante a da OECD. Contudo, algumas diferenças importantes são observadas entre as respectivas sociedades. Na Europa, a EMEA sugere a avaliação de rotina de parâmetros funcionais, como a produção de anticorpos dependente de células T, atividade de células naturak killer (NK), além da fenotipagem de subpopulações linfocíticas por citometria de fluxo. Nos Estados Unidos, a Food and Drug Administration (FDA) preconiza a realização de testes funcionais através de estudos caso-a-caso. Por sua vez, a sociedade japonesa Ministry of Healthy, Labour and Welfare (MHLW) sugere a fenotipagem de subpopulações linfocíticas e imunoistoquímica do baço de forma rotineira. Assim, caso algum efeito

imunomodulador seja observado durante o estudo de doses repetidas, testes funcionais são divididos em duas etapas subseqüentes (Tier 1 e Tier 2), cada uma dependente de resultados positivos obtidos na anterior (Putman et al., 2003).

Em uma revisão excelente, Dean (2004) desenvolve o seguinte argumento: "pathology alone, while adequate to identify many potential immunotoxic agents, can not with 100% confidence identify all immunologically active agents that might be identified in one or more functional screening tests". Isso significa que o paradigma ideal para a imunotoxicologia deveria consistir de parâmetros convencionais de toxicidade com alto poder de predição, como a análise histopatológica (Kuper et al., 2006), associados a um ou mais testes funcionais de elevada sensibilidade. Ainda, quando pensamos em substâncias com a capacidade de promover estímulo imunológico e o desenvolvimento de processos auto-imunes, caracterizados por uma etiologia complexa e multifatorial, o desafio de validar metodologias com bom poder de predição torna-se ainda maior.

1.4. Plantas medicinais com potencial imunomodulador

Desordens imunológicas têm um histórico notável de tratamento através do uso de plantas medicinais (Gertsch, 2008). Plantas imunomoduladoras possuem a capacidade de alterar o sistema imunológico através da regulação dinâmica de moléculas mensageiras tais como citocinas, moléculas de adesão, óxido nítrico, hormônios, neurotransmissores e outros peptídeos (Spelman et al., 2006).

Diversos estudos e revisões disponíveis na literatura científica sugerem que grande parte dos efeitos imunológicos ocasionados pelo uso de plantas medicinais possa se dever à modulação da produção de determinadas citocinas (Calixto et al., 2004).

Citocinas são peptídeos pleiotrópicos com múltiplas origens, alvos e funções biológicas. Sua atividade é vital durante processos inflamatórios desencadeados pela imunidade inata e adaptativa, assim como para o crescimento, diferenciação e morte celular, angiogênese e processos de reparo tecidual (Oppenheim, 2001). Assim, a modulação da atividade das citocinas pode se converter em opções terapêuticas para o tratamento de doenças como alergias, autoimunidade ou mesmo o câncer (Nelson e Ballow, 2003). Atualmente, as opções farmacológicas consistem no uso de anticorpos que antagonizam ou estimulam a atividade de determinadas citocinas através de interações com receptores celulares específicos ou então a administração de citocinas recombinantes (Spelman et al., 2006). Contudo, essas práticas podem ocasionar a incidência de efeitos adversos severos, criando barreiras para sua adoção clínica. Por exemplo, o uso das citocinas recombinantes IFN-γ, TNF-α e IL-2 pode provocar o desenvolvimento de linfopenia e neutrofilia transitórias (Lee et al., 2010). Dessa forma, produtos naturais podem ser uma boa opção terapêutica para o manejo de doenças da imunidade.

Plantas medicinais utilizadas tradicionalmente para o tratamento de desordens inflamatórias, alérgicas e autoimunes, atualmente são reconhecidas pela sua capacidade de modular a produção de citocinas e a diferenciação de linfócitos T rumo a um perfil Th1 ou Th2. Por exemplo, é sugerido que a espécie *Cyperi Rhizoma* estimula preferencialmente o desenvolvimento da linhagem Th1 através do estímulo da transcrição de IFN-γ e T-bet, enquanto a expressão de IL-4 e GATA-3 foi significantemente reduzida (Bae et al., 2010). Diferentes doses da fração alcalóide da espécie *Sinomenium acutum* resultou em uma redução da produção de mediadores da resposta Th1 (IgG2 e IFN-γ) e Th2 (IgG1, IgE e IL-5) (Feng et al., 2006). *Periploca sepium*, uma espécie tradicional chinesa, é capaz de inibir a proliferação de linfócitos T estimulados por mitógeno, além da expressaão de IL-2R, IFN-γ e IL-2, sendo utilizada

para o tratamento de doenças auto-imunes do tipo Th1, como a artrite reumatóide. A fórmula chinesa "Yi-fey Ruenn-hou", constituída por alcaçuz, ginseng, *Radix paeoniae alba* e chá verde, administrada por via oral a camundongos elevou os níveis séricos das citocinas Th1 (IL-2, IFN-γ) e, particularmente, Th2 (IL-4 e IL-10) de forma dose dependente (Lin et al., 2004).

Outro mecanismo muito interessante pelo qual produtos naturais podem modular o desenvolvimento de doenças autoimunes consiste na modulação de uma população de linfócitos com atividade imunossupressora conhecida como células T reguladoras (Tregs). A modulação da atividade das células Tregs pode ser realizada com dois objetivos distintos. Por um lado, o aumento do número e função das células Tregs pode ser promovido a fim de se suprimir uma resposta imunológica inadequada, como observado em condições inflamatórias e autoimunes (Vandenbark e Offner, 2008). Por exemplo, um estudo que utilizou a fração alcalóide da espécie *Sophora alopecuroides* revelou que a espécie promove um aumento do número de células Tregs produtoras de IL-10 em um modelo experimental de colite autoimune em ratos, resultando na redução do processo inflamatório e na melhoria dos sintomas (Zhou et al., 2010). O componente diosgenina, uma saponina esteroidal obtida a partir da espécie *Dioscorea nipponica*, apresentou a capacidade de estimular o número de células Tregs produtoras de IL-10 e, como conseqüência, inibir uma resposta do tipo Th2 em um modelo de alergia intestinal em camundongos BALB/c (Huang et al., 2010). Em um estudo realizado com voluntários obesos, o componente epigallocatequina-gallato encontrado na espécie *Camellia sinensis*, promoveu o aumento do número e função de células Tregs produtoras de IL-10 *in vitro,* sugerindo um possível benefício contra os processos inflamatórios observados em pessoas com sobrepeso (Yun et al., 2010). Estudos *in vitro* demonstram que extratos da espécie *Artemisia princeps* promovem a expansão de

células Tregs produtoras de IL-10, inibindo a proliferação de linfócitos T $CD4^+$ e a síntese das citocinas pró-inflamatórias IL-2 e IFN-γ (Chang et al., 2010). Em um modelo de artrite reumatóide autoimune induzida por colágeno, extratos da espécie *Chelidonium majus* promoveram o aumento do número de células Tregs e a redução das citocinas pró-inflamatórias TNF-α, IL-6, IFN-γ, *in vivo,* protegendo a cartilagem de camundongos DBA1/J contra a destruição (Lee et al., 2007). Por outro lado, a redução do número e da função das células Tregs pode ser a opção de escolha nos casos de neoplasias, situações nas quais uma atividade imunológica eficiente é necessária a fim de se evitar a progressão tumoral (Curiel et al., 2008). A injeção do diterpeno sclareol e do sesquiterpeno tehranolide no interior de um carcinoma ductal de mama em camundongos BALB/c resultou na redução do número de células Tregs imunossupressoras no microambiente tumoral. Uma vez restaurada a imunovigilância dos animais, o crescimento tumoral foi inibido (Noori et al., 2010). Estudos demonstram que a espécie *Curcuma longa* possui a capacidade de inibir a produção das citocinas IL-10 e TGF-β e a atividade supressora das células Tregs, promovendo um desvio imunológico para um padrão predominantemente Th1 e auxiliando na erradicação de células tumorais (Bhattacharyya et al., 2010). De forma semelhante, a espécie *Psidium guajava* inibiu a proliferação de células de melanoma injetadas em camundongos através da redução da atividade imunossupressora das células Tregs e da promoção de uma resposta imunológica do tipo Th1 (Seo et al., 2005).

Segundo os exemplos citados acima, fica claro o potencial de produtos naturais em modular aspectos distintos do sistema imunológico. Nesse contexto, duas espécies vegetais se destacam pela sua atividade antiinflamatória e imunomoduladora. Conhecidas popularmente como “unha-de-gato”, as espécies *Uncaria tomentosa e Uncaria guianensis* apresentam um longo historio de uso tradicional destinado ao

tratamento de desordens imunológicas. A seguir, abordaremos com maior detalhe os aspectos mais relevantes dessas plantas.

1.4.1. *Uncaria tomentosa* e *Uncaria guianensis*

Uncaria tomentosa e Uncaria guianensis (Rubiaceae) são espécies nativas da floresta Amazônica e amplamente utilizadas pela medicina tradicional para o tratamento de doenças como o diabetes, câncer, infecções intestinais e desordens inflamatórias (Valério e Gonzales, 2005). Até o momento, sua caracterização fitoquímica revelou a presença de alcalóides indólicos e oxindólicos (planta toda) (Laus e Keplinger, 1994), proantocianidinas e glicosídeos triterpenóides (casca) (Sandoval et al., 2000; Yépez et al., 1991) e flavonóides (casca e folhas) (Valente et al., 2009).

O potencial da espécie *U. tomentosa* em modular o sistema imunológico vem sendo descrito por alguns estudos científicos, contudo os resultados variam consideravelmente segundo as condições experimentais utilizadas. Extratos obtidos a partir da casca da *U. tomentosa* inibiram a expressão de TNF-α, iNOS and NF-κB, além de aumentar a síntese de IL-1β e IL-6, estimular a fagocitose e elevar o número absoluto de leucócitos periféricos (Akesson et al., 2003; Allen Hall et al., 2007; Lemaire et al., 1999; Sandoval et al., 2000; Wagner et al., 1985; Wurm et al., 1998). O potencial imunomodulador da espécie também foi demonstrado através de ensaios de proliferação celular. O extrato etanólico e a fração alcalóide da *U. tomentosa* inibiram a proliferação de células mononucleares humanas estimuladas pelos mitógenos fitohemaglutinina (PHA) e concanavalina A (Con A) *in vitro* (Winkler et al., 2003). De forma semelhante, o extrato comercial aquoso resultou na inibição da proliferação de linfócitos T e B, sem interferir com a produção da citocina IL-2 *in vitro* (Akesson et al., 2003). Contudo, apesar da predominância de efeitos antiproliferativos, ratos tratados *in*

vivo com esse mesmo extrato comercial aquoso apresentaram um aumento da taxa proliferação de linfócitos estimulados pelo mitógeno PHA (Sheng et al., 2000).

Na década de 1980, pesquisadores da Universidade de Munique, Alemanha, demonstraram que o aumento da atividade fagocítica de granulócitos ocorre após o tratamento com extratos de *U. tomentosa* com alto teor de alcalóides. Nas condições experimentais propostas, a fagocitose foi potencializada pelo alcalóide pentacíclico pteropodina, contudo, os alcalóides tetracíclicos isomitrafilina e isorinchofilina não apresentaram efeitos significativos (Wagner et al., 1985). Posteriormente, Wurm et al. (1998) revelaram que uma mistura de alcalóides pentacíclicos (especiofilina, mitrafilina, uncarina F, isomitrafilina, pteropodina e isopteropodina) tem o potencial de induzir células endoteliais a liberarem determinado(s) fator(s), ainda não desvendado(s), promovendo uma significativa melhora da resposta imunológica. A partir desses estudos, foi estabelecido que a eficácia do material vegetal e dos fitoterápicos derivados da *U. tomentosa* seria dependente da concentração e do perfil desses alcalóides.

Modelos *in vitro* demonstram que a espécie *U. tomentosa* inibe de maneira dose dependente a expressão do fator de transcrição NF-kB e a produção de TNF-α em cultura de células RAW 264.7 estimuladas por LPS. Esse mesmo estudo relatou uma atividade antioxidante eficiente, prevenindo a formação de radicais livres e a necrose de células RAW 264.7 expostas a 2,2-difenil-1-picrilhidrazil (DPPH) e a radiação ultra-violeta (UV) (Sandoval, 2000).

Aquino et al. (1991) demonstraram a atividade antiviral de 6 glicosídeos do ácido quinovínico derivados do extrato de *U. tomentosa*. Sua eficácia foi avaliada contra duas infeções por RNA vírus (vírus da estomatite vesicular – VEV- e rinovírus 1B), em linhagens de células CER e HeLa, respectivamente. Um efeito inibitório em

casos de infecção pelo VEV foi observado para os 6 glicosídeos estudados. Em contraste, apenas um composto (ácido quinovínico B-D-glucopiranosil-28→-B-D-glucopiranosil éster) demonstrou atividade inibitória contra o rinovírus 1B. Recentemente, pesquisadores do nosso grupo revelaram a atividade antiviral da fração alcalóide da *U. tomentosa*, resultando em uma inibição da infecção de monócitos humanos pelo vírus da dengue do tipo 2, seguida pela redução da produção de IFN-γ e TNF- α (Reis et al., 2008).

Até o momento, alguns estudos clínicos foram conduzidos com extratos de *U, tomentosa.* Treze pacientes portadores do HIV tratados durante 5 meses com cápsulas do extrato hidroetanólico de *U. tomentosa* contendo 12 mg de alcalóides pentacíclicos apresentaram um aumento da contagem relativa e absoluta de linfócitos (Keplinger, 1989). Contudo, esse estudo possui deficiências por não contar com um grupo controle e o acompanhamento de outros fatores diagnósticos durante o período experimental. Em outros dois estudos clínicos, 12 indivíduos tratados com o extrato aquoso da *U. tomentosa* na dose de 5 mg/kg apresentaram um discreto, porém significativo, aumento da contagem de leucócitos após 6 semanas e o aumento da capacidade de reparo do DNA após dano induzido por peróxido de hidrogênio, ao final de 8 semanas (Sheng et al., 2000). No entanto, estudos adicionais devem ser realizados para se criar uma curva dose-resposta adequada, além de avaliar os seus efeitos sobre grupos populacionais diversos.

Estudos toxicológicos reportam uma baixa incidência de toxicidade para a espécie *U. tomentosa.* Sheng et al. (2000) sugere que a DL50 (dose letal 50) do extrato aquoso seja superior a 8 g/kg, além de reportar a ausência de sinais significativos de toxicidade sistêmica após o tratamento com o extrato (Sheng, 2000). Contudo, as condições experimentais desse estudo foram limitadas a ratos Wistar-Furth fêmeas e a

um regime de baixas doses. Em outro estudo, ratos Wistar tratados com um extrato de *U. tomentosa* contendo uma concentração de alcalóides totais de 7.5 mg/g apresentaram linfocitose e neutropenia, além do aumento do peso dos rins (Svendsen and Skydsgaard, 1986 – unpublished data). No entanto, os resultados do estudo foram restritos a um extrato aquoso e não focaram em uma relação dose-resposta. O número de informações disponíveis a respeito da incidência de efeitos adversos resultantes da exposição aguda ou crônica de humanos aos extratos de *U. tomentosa* é limitado, contudo um caso foi relatado descrevendo um paciente com lúpus eritematoso sistêmico que desenvolveu insuficiência renal associada à ingestão do extrato (Valério e Gonzales, 2005).

Ao contrário da espécie *U. tomentosa,* poucos estudos estão disponíveis na literatura a respeito do potencial terapêutico da espécie *U. guianensis*. Sandoval et al. (2000) demonstraram que o extrato aquoso da casca da *U. guianensis* é capaz de inibir a produção de radicais livres e a peroxidação de lipídios, além de reduzir a produção de TNF-α induzida por mitógeno *in vitro.* Em outro estudo, Carvalho et al. (2006) revelaram uma efetiva atividade antiinflamatória e antialérgica, *in vivo* e *in vitro.* Nesse estudo, o extrato etanólico das folhas da *U. guianensis* reduziu o edema da pata de camundongos e o exudato pleural induzidos por zimozan ou ovalbumina, além de inibir a produção da citocina IL-5, óxido nítrico e CXCL8 por macrófagos peritoneais.

Alguns estudos clínicos buscaram explorar a atividade antiinflamatória da espécie *U. guianensis.* Piscoya et al. (2001) demonstraram o potencial do extrato aquoso da casca da *U. guianensis* para o tratamento de pacientes apresentando artrite nos joelhos através da inibição da produção de TNF-α e da via de sinalização do fator de transcrição NF-κB. Além disso, um extrato comercial contendo uma associação de *U. guianensis e Lepidium meyenii* resultou em um potente efeito protetor para as cartilagens de pacientes portadores de artrite, estimulando o fator de crescimento do tipo

insulina (IGF-1) e prevenindo a atividade catabólica da IL-1ß (Miller, 2006). Até o momento, nenhum estudo relativo à toxicidade da espécie *U. guianensis* está disponível na literatura científica.

Uma vez discutido o potencial de ambas as espécies, *U. tomentosa* e *U. guianensis,* modularem parâmetros imunológicos, seu uso pode se tornar uma interessante ferramenta para a execução de estudos de imunomodulação de doenças autoimunes, tal qual o diabetes melito 1 (DM1).

1.5. Diabetes melito do tipo 1

1.5.1. Fatores de susceptibilidade ao desenvolvimento do diabetes melito do tipo 1

O diabetes melito do tipo 1 (DM1) é uma doença autoimune crônica caracterizada por um infiltrado mononuclear inflamatório nas ilhotas pancreáticas e a conseqüente destruição das células β produtoras de insulina. Uma vez que o processo autoimune seja iniciado, uma fase assintomática inicia-se, podendo ser monitorada através da presença de autoanticorpos séricos (Anderson e Bluestone, 2005; Eisenbarth, 2004). O desenvolvimento do DM1 é geneticamente controlado e acredita-se que fatores ambientais, tais como infecções virais, possam ser o gatilho necessário para o início e a progressão da doença (von Herrath, 2009). Estima-se que cerca de 400 em cada 100000 crianças americanas nascem em famílias com histórico da doença (Dabelea et al., 2007). Esse grupo específico apresenta um risco de desenvolver o DM1 excedendo 5%, quando comparado aos 0.4% dos indivíduos sem histórico familiar da doença. Esse risco pode ser ainda mais significativo se o estratificarmos com base no membro familiar afetado, correspondendo a 3%, 5% ou 8% para mãe, pai e irmão afetados, respectivamente. Em raras situações, no caso de dois membros familiares diretos afetados, esse risco sobe

para 20% (Bonifacio et al., 2004; Hemminki et al., 2009). Foi demonstrado que a susceptibilidade genética ao desenvolvimento do DM1 correlaciona-se diretamente com determinados genótipos do antígeno leucocitário humano (HLA). Em virtude de suas múltiplas funções durante a seleção de células T, apresentação de antígenos e desenvolvimento da resposta imunológica, o HLA influencia diretamente o tipo de antígenos reconhecidos pelos linfócitos T autoreativos, sendo considerado como o primeiro "checkpoint" para o desenvolvimento da autoimunidade (Ziegler e Nepom, 2010). Estudos demonstram os alelos HLA DRB1 *03, *04 e HLA DQB1 *03,*02 classe II como os genótipos de maior risco para o desenvolvimento do DM1, estando presente em 2,3% das crianças caucasianas dos Estados Unidos e em 39% dos pacientes que desenvolvem a doença antes dos 20 anos de idade (Lambert et al., 2004).

1.5.2. Papel das células T na patogenia do diabetes melito do tipo 1

Baseado em diversos modelos experimentais de DM1 espontâneo, tais como o camundongo não-obeso diabético (NOD) e o rato Biobreeding (BB), os mediadores primários da destruição das células-β parecem ser os linfócitos T $CD4^+$ e $CD8^+$. Esses linfócitos T patogênicos exibem tipicamente um fenótico do tipo Th1, caracterizado pela produção de citocinas inflamatórias como o IFN-γ e TNF-α (Anderson e Bluestone, 2005; Eisenbarth, 2004). Apesar de diversas linhagens de linfócitos Th terem sido identificadas, duas subpopulações, conhecidas como T-helper 1 (Th1) e T-helper 2 (Th2), estão melhor identificadas com relação ao seu papel fisiológico e sua atuação em determinadas doenças, tais como processos inflamatórios e autoimunes. A diferenciação e a função das células Th1 e Th2 são reguladas por uma complexa rede de citocinas e fatores de transcrição. A ativação de células T $CD4^+$ *in vitro* na presença de IL-12

promove a ativação dos fatores de transcrição STAT4 e T-bet, os quais por um lado estimulam a expressão do gene IFN-γ e a diferenciação Th1, e por outro silenciam a expressão da IL-4, GATA3 e inibem a diferenciação Th2 (Thieu et al., 2008; Ansel et al., 2006). Por sua vez, a ativação de células T $CD4^+$ *in vitro* pela IL-4 promove a diferenciação Th2 através ativação do fator de transcrição STAT6 e GATA3, os quais por sua vez inibem a expressão de IL-12 e STAT 4 e a diferenciação Th1 (Ansel et al., 2006). Contudo, é importante salientar que a caracterização de linhagens de linfócitos Th apresenta algumas limitações, visto que uma divisão nem sempre é clara em doenças humanas.

Estudos em camundongos NOD revelaram que o DM1 pode ser transferido para recipientes singênicos imunocomprometidos através da doação de linfócitos T $CD4^+$ e $CD8^+$, mas não pela transferência de cada subtipo celular separadamente (Phillips et al., 2009). A capacidade de anticorpos do tipo anti-CD3 em reverter o DM1 em camundongos NOD também colabora para salientar o papel fundamental dos linfócitos T para a patogenia da doença. Nesse sentido, alguns estudos clínicos foram conduzidos utilizando-se anticorpos monoclonais anti-CD3 em pacientes com DM1 em estágio inicial, demonstrando que o bloqueio resulta na supressão da destruição das células β pancreáticas (Chatenoud, 2010).

Atualmente, acredita-se que diversos mecanismos atuam conjuntamente para a destruição das células β. Células T $CD8^+$ são capazes de promover a destruição das celulas através de citotoxicidade direta mediada pelo complexo principal de histocompatibilidade do tipo 1 (MHC I) (Scott et al., 2010). Além disso, a produção de IFN-γ pelas células T $CD4^+$ e $CD8^+$ induz a expressão do receptor de morte célular FAS o qual, uma vez complexado ao FAS-ligante das células T efetoras, pode iniciar o processo de apoptose das células β (Kim e Lee, 2009). Adicionalmente, a produção de

IFN-γ promove a ativação de macrófagos e regula positivamente a expressão de citocinas pró-inflamatórias como IL-1β e TNF-α, além de induzir a produção de espécies reativas de oxigênio (ROS), fornecendo assim um segundo estímulo para a destruição celular (Kim e Lee, 2009).

1.5.3. Papel das células B na patogenia do diabetes melito do tipo 1

Até o momento alguns autoanticorpos específicos para antígenos das ilhotas pancreáticas foram identificados, destacando-se anticorpos contra insulina e pró-insulina (Mohan et al., 2010), GAD65 e GAD67 (Ou et al., 2000), IA-2 (Weenink et al., 2009) e ZnT8 (Yang et al., 2010). A detecção de apenas um tipo de autoanticorpo, entre todos os citados anteriormente, não representa um risco significantemente maior de desenvolvimento do DM1, podendo estar presente tanto em indivíduos com ou sem histórico familiar da doença. Contudo, a presença de dois ou mais autoanticorpos específicos para antígenos das ilhotas está diretamente relacionado ao aumento do risco do desenvolvimento da doença, assim como sua severidade (Taplin e Barker, 2008).

Atualmente, acredita-se que linfócitos B estão implicados no desenvolvimento de processos autoimunes através de sua capacidade de atuar como células apresentadoras de antígeno (APCs). A depleção de linfócitos B em camundongos NOD inibe o desenvolvimento do DM1, sugerindo seu papel para a patogenia da doença (Smith e Tedder, 2009). Confirmando esse achado, a depleção de células B em pacientes portadores do DM1 através da administração do anticorpo monoclonal rituximab resultou em uma melhora da função e das células β pancreáticas (Pescovitz et al., 2009).

1.5.4. Mecanismos da imunidade inata envolvidos na patogenia do diabetes melito do tipo 1

Estudos experimentais demonstram a presença de macrófagos no interior das ilhotas de camundongos NOD, assim como seu papel deletério através da produção de mediadores pró-inflamatórios como a IL-1β, TNF-α e ROS. Macrófagos ativados já foram descritos nas ilhotas pancreáticas de camundongos NOD antes mesmo da ocorrência do infiltrado de células T, assim como em camundongos NOD imunocomprometidos (NOD/Scid), os quais não apresentam células T e B funcionais (Arnush et al., 1998). Outro mecanismo efetor pelo qual os macrófagos parecem contribuir para a progressão do DM1 ocorre através da síntese de IL-12, promovendo a diferenciação de linfócitos T $CD8^+$ diabetogênicos (Yoon e Jun, 1999). Estudos revelam que o bloqueio da migração de macrófagos para as ilhotas de camundongos NOD é capaz de inibir o desenvolvimento do DM1 (Hutchings et al., 1990).Tais achados apontam para o papel desse grupo celular como reguladores da resposta imunológica no modelo NOD.

Células "natural-killer" (NK) também podem ser observadas no interior das ilhotas de camundongos NOD e NOD-rag (ausência de células T e B maduras), apresentando um fenótipo ativado caracterizado por uma elevada produção de IFN-γ (Gur et al., 2010). Estudos em diversos modelos experimentais revelaram que o bloqueio das células NK por anticorpos monoclonais previne o desenvolvimento do DM1, sugerindo um papel importante na mediação da destruição das células β (Alba et al., 2008).

Em condições fisiológicas, células dendríticas (DCs) são capazes de induzir tolerância periférica, através da deleção ou anergia de linfócitos T autoreativos ou por

meio da expansão de linfócitos Tregs (Ueno et al., 2007). Contudo, alguns estudos demonstram que células dendríticas (DCs) convencionais de camundongos NOD podem contribuir para o processo autoimune através da produção de grandes quantidades de IL-12 e da expressão de moléculas co-estimulatórias (Steptoe et al., 2002). Estudos adicionais sugerem que DCs plasmocitóides de camundongos NOD, produtoras de IFN do tipo I, podem em alguns casos induzirem ou potencializarem a progressão do DM1 (Li et al., 2008).

1.5.5. O papel das células T reguladoras na saúde e na doença

Como discutido anteriormente, a imunidade adaptativa requer um delicado controle entre os mecanismos de tolerância a antígenos próprios e, simultaneamente, a capacidade de desencadear uma resposta efetora direcionado a uma ampla gama de patógenos infecciosos.

Atualmente, existe uma ampla evidência sobre o potencial das Tregs em suprimir a atividade de linfócitos efetores autoreativos periféricos, assim como o início do processo autoimune (Sakaguchi et al., 2010). Na década de 90, Sakaguchi et al. (1996) demonstraram que uma subpopulação de linfócitos T $CD4^+$ de roedores possuía uma potente atividade supressora, *in vitro* e *in vivo*. De forma simplificada, as células Tregs são classificadas como naturais ou induzidas (Le e Chao,2007). Células Tregs naturais (nTregs) desenvolvem-se no timo, sendo positivamente selecionadas pelas células epiteliais corticais caso seus receptores de células T (TCRs) apresentem uma afinidade intermediária à peptídeos-próprios (Bensinger et al., 2001). Uma vez selecionadas, as nTregs direcionam-se para a periferia, local no qual exercerão um controle dominante sobre linfócitos T autoreativos (Baecher-Allan et al., 2004). Células

nTregs expressam constitutivamente uma série de moléculas de superfície relacionas com fenótipos de ativação e memória, incluindo a cadeia α do receptor para IL-2 (CD25), $CD45RB^{low}$, CD62L, CTLA-4, GITR e FoxP3. (Le e Chao; Ziegler, 2006). Em contrates com o desenvolvimento tímico das nTregs, células Tregs induzidas (iTregs) são geradas na periferia, possivelmente com o objetivo de finalizar a resposta imunológica uma vez que determinado patógeno tenha sido eliminado. Sua geração ocorre a partir de linfócitos T naïve e depende de estímulos tais como a sinalização fornecida pelas células APCs (Jonuleit et al., 2000), atividade de citocinas como o TGF-β (Chen et al., 2003) e a presença de baixas concentrações de um antígeno (Apostolou et al., 2002). Existem duas subpopulações distintas de células iTregs cujo fenótipo é definido principalmente pelo tipo de citocina predominantemente produzida: células Th3, caracterizadas pela produção de TGF-β, e Tr1, caracterizadas pela produção de IL-10 (Le e Chao, 2007)

Estudos demonstram que as Tregs representam apenas 5-10% das células T $CD4^+$ e cerca de 3% dos linfócitos totais periféricos (Ziegler, 2006). Dessa forma, uma migração direcionada especificamente para os locais de estímulo e uma expansão antígeno-específica são necessários para uma resposta supressora eficiente. Esse dado é ilustrado através de estudos em camundongos NOD, revelando que uma atividade supressora e a prevenção do DM1 podem ser obtidas apenas se as Tregs forem capazes de migrar e proliferar nos linfonodos pancreáticos após sua transferência ao animal (Jaeckel et al., 2005).

Contudo, os mecanismos pelos quais as Tregs exercem supressão são ardilosos, com diferenças observadas entre os resultados *in vivo* e *in vitro.* Diversos estudos *in vitro* sugerem que as nTregs suprimem a atividade de linfócitos T efetores segundo mecanismos dependentes de contato celular, em particular através das moléculas de

superfície CTLA-4 e GITR, mas independentes da sinalização por citocinas (Takahashi et al., 2000; Piccirillo et al., 2002). Já as iTregs induzidas (Th3 e Tr1) parecem exercer sua atividade supressora através da secreção de citocinas tais como IL-10 (Hara et al., 2001) e TGF-β (Josien et al., 1998). Atualmente, esforços são concentrados no sentido de buscar uma correlação entre os mecanismos de supressão por contato celular e sinalização por citocinas.

1.6. Modelos experimentais para o estudo do diabetes melito do tipo 1

Uma das primeiras dificuldades enfrentadas durante o uso de modelos animais para o estudo do DM1 é a falta de conhecimento sobre os exatos mecanismos envolvidos na doença humana, incluindo qual antígeno específico das células β inicia o processo autoimune, a extensa heterogeneidade entre os casos, a relação entre infecções virais e o DM1 e os mecanismos pelos quais as células β morrem (Akirav et al., 2008). Além disso, diversas vias de sinalização redundantes contribuem para a progressão do DM1, tornando difícil a escolha de um único alvo terapêutico para o manejo da doença.

O modelo de DM1 espontâneo mais estudado consiste na espécie NOD. Estudos genéticos realizados durante os últimos 25 anos com essa linhagem de camundongos demonstraram múltiplos *loci* de susceptibilidade e, assim como em humanos, a região codificadora MHC II (molécula I-A^{g7}) apresenta um papel chave (Li et al., 2009). Contudo, um único *locus* não é necessário nem suficiente para o desenvolvimento do DM1 nessa espécie, uma vez que foram descritos *loci* não-MHC de susceptibilidade à doença, incluindo regiões que codificam a IL-2 e CTLA-4. Esses resultados colaboraram para demonstrar que múltiplas vias de susceptibilidade genética são

necessárias para o desenvolvimento do diabetes melito autoimune, ao menos no modelo experimental NOD (Ilonen e Hermann, 2010).

Outro modelo extensivamente utilizado que contribuiu para o estudo dos mecanismos envolvidos no DM1 envolve a administração de múltiplas doses subdiabetôgenicas de estreptozotocina (MLDS) a algumas linhagens de camundongos, com ênfase para a espécie C57BL/6. A estreptozotocina (STZ), originalmente isolada do *Streptomyces achromogenes,* é uma nitrosurea (N-methyl-N-nitrosurea) que apresenta atividade antibiótica, antineoplásica e diabetogênica (Bolzan e Bianchi, 2005). Sua absorção pelas células ß das ilhotas pancreáticas se processa através da membrana celular pelo transportador de glicose GLUT2 (Tjälve et al.,1976; Karunanayake et al., 1976). Dessa forma, células produtoras de insulina que não expressam esse transportador são resistentes à ação da STZ. A importância do GLUT2 evidencia-se ainda através da observação de que a STZ exerce toxicidade em outros órgãos que expressam o transportador, particularmente o fígado e os rins (Weiss et al., 1982). Uma série de estudos demonstrou que a indução do diabetes em roedores pela STZ pode proceder por duas vias distintas: i) a injeção de uma única dose elevada de STZ (100-200 mg/kg), causando uma rápida destruição das células ß pancreáticas por apoptose ou necrose e hiperglicemia sustentada, mesmo em camundongos imunodeficientes. ii) a administração de MLDS (40 mg/kg), ocasionando a geração de espécies reativas de oxigênio, induzindo a expressão do autoantígeno descarboxilase do ácido glutâmico (GAD) pelas células β e, finalmente, o desenvolvimento da insulite (Like e Rossini, 1978; Elias et al., 1994).

Estudos defendem que o modelo de DM1 induzido por MLDS representa uma doença autoimune dependente de células T. Contudo, diversos mecanismos parecem

atuar conjuntamente, incluindo a atividade de células T $CD4^+$ e $CD8^+$, células B, macrófagos e a produção de espécies reativas de oxigênio.

No modelo de DM1 induzido por MLDS, o aumento de citocinas próinflamatórias padrão Th1 e a redução da produção de citocinas do tipo Th2 estão diretamente relacionados com o desenvolvimento e progressão da doença em camundongos C57BL/6. Em função da ativação da cascata inflamatória, espécies reativas de oxigênio (ROS) também foram implicadas como importantes mediadoras da destruição de células ß pancreáticas nesse modelo experimental (Muller et al., 2002). Macrófagos parecem ser as primeiras células a infiltrar as ilhotas de camundongos tratados com MLDS, atuando tanto na apresentação de antígenos como causando danos e disfunção das células β através da síntese de radicais reativos de oxigênio. A deleção de genes que codificam a galectina-3, uma lectina responsável por regular positivamente a migração e a atividade de macrófagos/monócitos, parece estar relacionada com uma atenuação do infiltrado mononuclear inflamatório das ilhotas pancreáticas, protegendo-as contra a destruição (Mensah-Brown et al., 2009). Em outro estudo, camundongos "knockout" para o fator inibitório de migração de macrófagos (MIF -/-), uma citocina pleiotrópica cujos níveis encontram-se elevados em algumas condições autoimunes, apresentaram proteção contra o desenvolvimento de insulite e contra a apoptose de células β induzidas por MLDS. Esses resultados foram acompanhados pela inibição da capacidade funcional dos macrófagos, e um desvio imunológico para um padrão do tipo Th2 (Stosic-Grujicic et al., 2008). Cantazaro et al. (2010) demonstraram que o receptor de bradicinina B1 torna-se mais expresso na superfície de macrófagos após o tratamento com MLDS, induzindo sua ativação, a liberação de radicais reativos de oxigênio e culminando com a apoptose das células β (Cantazaro et al., 2010).

Na tentativa de elucidar os mecanismos imunológicos envolvidos no DM1 induzido por MLDS, foram propostas duas vias: a) uma toxicidade direta direcionada à molécula GLUT2 das células ß; b) estimulação da produção de IFN-γ e TNF-α dependente de células T e a ativação de fatores de transcrição inflamatórios tais como NF-κB.

Diversas evidências sugerem linfócitos T como mediadores do processo autoimune no modelo MLDS. Anticorpos direcionados contra moléculas da superfície de linfócitos, tais como CD3 (Herold et al., 1992), CD4 e CD8 (Kantwerk et al., 1987; Oschilewski et al., 1986) Thy-1 (Oschilewski et al., 1986), TCR $V_ß8$ (Herold et al., 1995), IL-2R (Hatamori et al., 1990), MHC classe II H-2A e H-2E (Kiesel et al., 1989) e LFA-1 (Herold et al., 1994), resultaram na inibição da insulite após o tratamento com MLDS. Estudos clássicos também sugeriram a participação de linfócitos T como mediadores do DM1 induzido por MLDS, demonstrando a capacidade de diversos agentes imunossupressores, tais como a ciclosporina e a azatioprina, em inibir o desenvolvimento da doença (Kolb et al., 1986; Yanagawa et al., 1989). Um estudo conduzido por Kiesel et al. (1980) demonstrou que camundongos C57BL/6 atímicos (nu/nu) que receberam esplenócitos de camundongos C57BL/6 tratados com MLDS desenvolveram insulite após um período de 14 dias. Apesar de suas limitações, esse estudo já apontava a importância dos linfócitos T $CD8^+$ e da restrição ao MHC classe I durante o processo de destruição das células ß. Em outro estudo clássico, Paik et al. (1980) fornecem evidências adicionais sobre o papel das células T no desenvolvimento do DM1 induzido por MLDS, uma vez que a doença ocorre apenas camundongos imunocompetentes e que a habilidade dos seus esplenócitos em transferir a autoimunidade a camundongos atímicos é inibida pela depleção de linfócitos T, mas não linfócitos B. Apesar de algumas evidências sugerirem que os linfócitos B não são

necessários para o início do processo autoimune no modelo MLDS, eles parecem influenciar diretamente a proliferação de clones de linfócitos T autoreativos necessária para a progressão da insulite, seja través da apresentação de antígeno ou por meio de sinais co-estimulatórios (Kondo et al., 2000)

Atualmente, é reconhecido que a ativação de linfócitos T naïve se processa não apenas através da interação ente o receptor de linfócitos T (TCR) e o MHC, mas também por meio de moléculas co-estimulatórias encontradas nas APCs (ex: CD80) e seu respectivo ligante nas células T (ex: CD28) (Janeway et al., 1994). Camundongos transgênicos que expressam a molécula CD80 (B7-1) na superfície de células ß pancreáticas apresentaram uma maior susceptibilidade ao desenvolvimento do DM1 após MLDS (Harlan et al., 1995). Esse mesmo estudo reforça o papel dos linfócitos T citotóxicos na patogênese do DM1 induzido por MLDS, uma vez que a doença foi prevenida pela depleção de células T $CD8^+$ e o bloqueio da molécula CD80. Em um estudo posterior, camundongos knockout cujos linfócitos não expressavam a molécula CD28 (CD28 -/-) apresentaram resistência ao desenvolvimento do DM1 induzido por MLDS, mesmo que as células ß pancreática expressassem a molécula co-estimulatória CD80 (B7-1). Em adição, a expressão da molécula CD80 (B7-1) pelas células ß pancreáticas foi relacionada com uma maior proporção de células T $CD8^+$ autoantígeno-específicas dispersas no infiltrado inflamatório das ilhotas (Pechhold et al., 2001). Atualmente, acredita-se que a destruição das células ß mediadas por linfócitos T $CD8^+$ tendem a ser antígeno-específicas, enquanto as células T $CD4^+$ agem principalmente favorecendo um microambiente inflamatório (Th1) (Wegmann et al., 1996).

Lgssiar et al. (2004) demonstraram que as ilhotas de camundongos C57BL/6 machos tratados com MLDS apresentaram uma produção de citocinas com um padrão tipicamente Th1, caracterizada pela redução da produção de IL-4, IL-10 e TGF-ß e um

aumento dos níveis de TNF-α e IFN-γ *ex vivo*. Esse mesmo estudo revelou que camundongos C57BL/6 machos tratados com MLDS e com a citocina antiinflamatória IL-11 apresentaram um desvio imunológico rumo a um padrão do tipicamente Th2, caracterizado pela redução das citocinas TNF-α e IFN-γ e pelo aumento dos níveis de IL-4, IL-10 e TGF-ß. Nesse caso, foi contatado que o tratamento com a IL-11 inibiu a ativação de fatores de transcrição próinflamatório, tais como o NF-κB, IKK-α e AP-1. Ao contrário dos machos, camundongos C57BL/6 fêmeas, naturalmente resistentes à indução do DM1, apresentam um aumento dos níveis de TNF-α e IFN-γ após MLDS, contudo os níveis de IL-4, IL-10 e TGF-ß não foram afetados pelo tratamento (Muller et al., 2002).

Finalmente, Zdravkovic et al. (2008) forneceram as primeiras evidências sobre o envolvimento das céluas Tregs FoxP3$^+$ na susceptibilidade de camundongos C57BL/6 ao regime de MLDS. Nesse estudo foi demonstrado que além do comprometimento da atividade das células Tregs, a deleção do gene que codifica a molécula ST2 resulta em uma polarização imunológica Th1, seguida pelo aumento da produção de IFN-γ e TNF-α e o desenvolvimento de insulite severa.

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Objetivos

2. Objetivos

Intervenções terapêuticas para o diabetes DM1 deveriam ser iniciadas antes do início da doença, ou pelo menos durante o período no qual um número razoável de células β produtoras de insulina encontram-se presente no pâncreas. Nesse contexto, o presente estudo foi delineado com o objetivo de avaliar o potencial das espécies vegetais *Uncaria tomentosa* e *Uncaria guianensis* em prevenir ou controlar a progressão do DM1 em camundongos C57BL/6. A pesquisa dividiu-se nas seguintes etapas:

1. ***Avaliação da imunotoxicidade dos extratos de U. Tomentosa e U. Guianensis:*** Camundongos BALB/c foram tratados com extratos de *U. tomentosa* e *U. guianensis* segundo normas estabelecidas pelo Guideline 407 da OECD (Organização para a Cooperação e Desenvolvimento Econômico). Foram avaliados parâmetros gerais de toxicidade, histologia de órgãos linfohematopoiéticos, fígado e rins; fenotipagem de subpopulações de linfócitos T e B, viabilidade e proliferação de linfóticos T e produção de citocinas padrão Th1 (IL-2, TNF-α, IFN-γ) e Th2 (IL-4, IL-5) (Figura i).

2. Imunomodulação do diabetes melito auto-imune: Os extratos de *U. tomentosa* e *U. guianensis* foram fornecidos a camundongos C57BL/6 diabéticos, avaliando-se glicemia semanal, parâmetros hematológicos, histológia dos órgãos linfohematopoiéticos, pâncreas, rins e fígado; imunoistoquímica de cortes do pâncreas para detecção de insulina, fenotipagem de linfócitos T e linfócitos Tregs e, finalmente, a dosagem de citocinas padrão Th1 (IL-2, TNF-α, IFN-γ) e Th2 (IL-4, IL-5) (Figura ii).

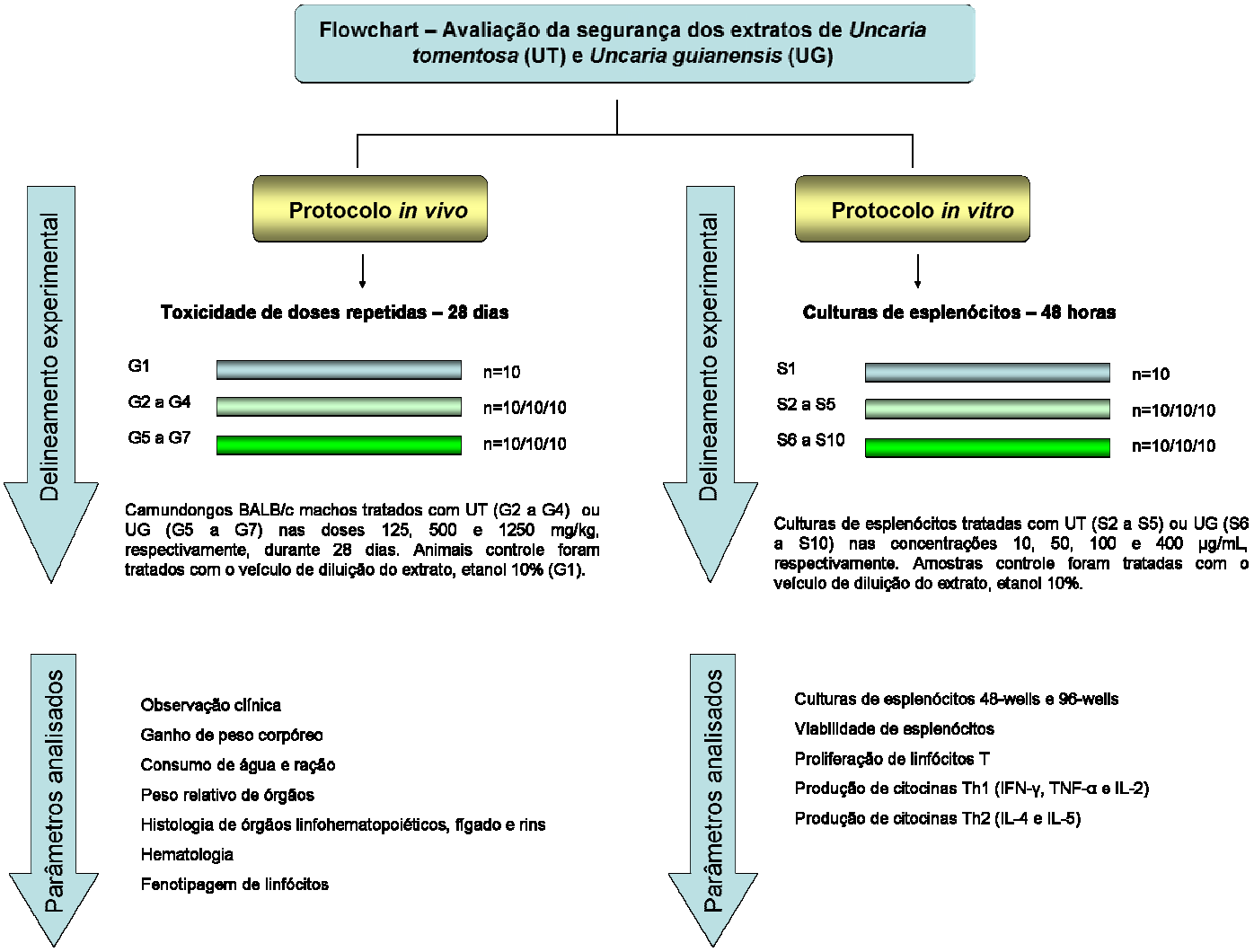

Figura i – "Avaliação do potencial imunotóxico dos extratos de *U. tomentosa* e *U. guianensis*". O protocolo experimental foi dividido em dois períodos distintos, objetivando-se caracterizar *in vivo* e *in vitro* o potencial imunotóxico e o efeito imunomodulador dos extratos.

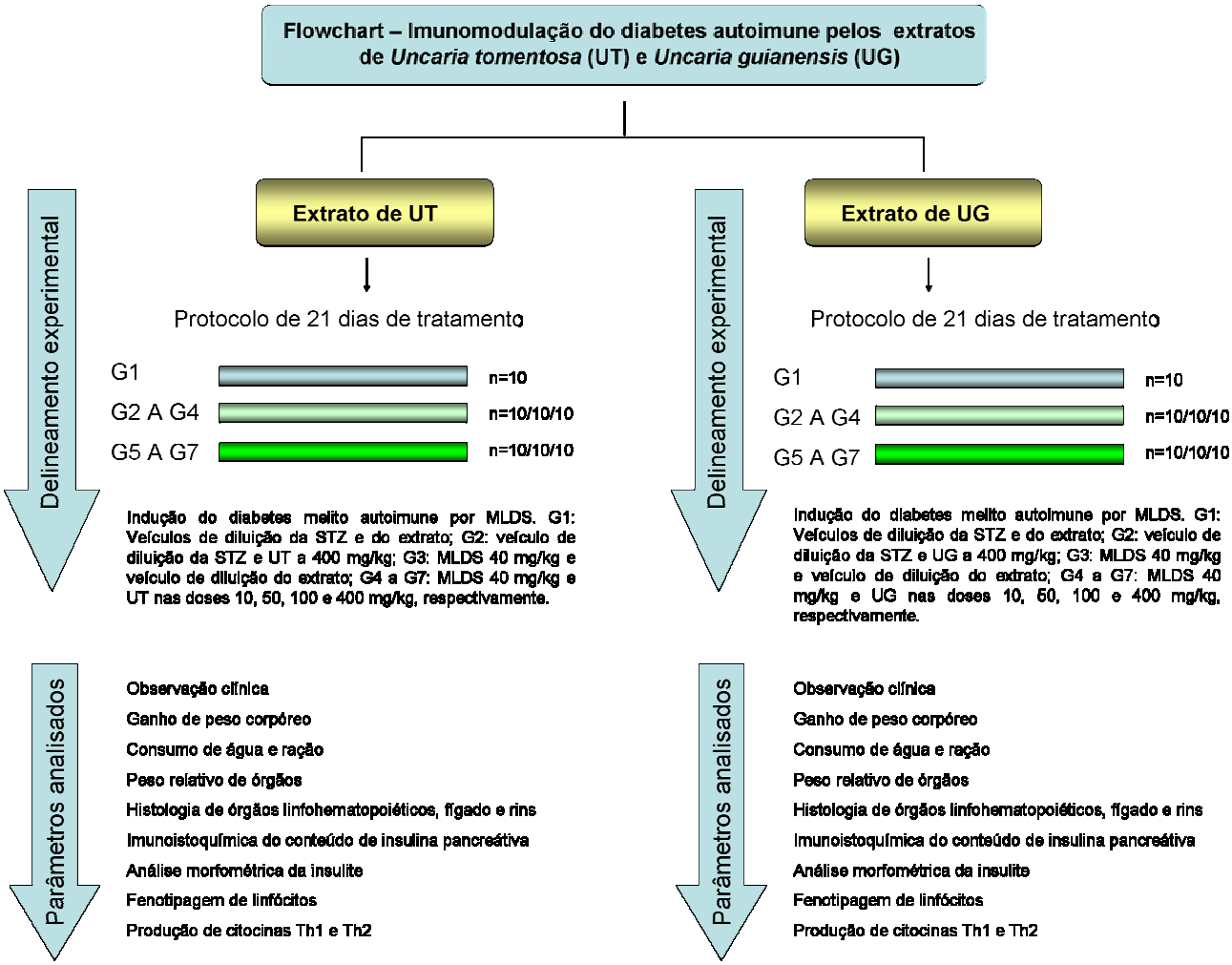

Figura ii – "Imunomodulação do diabetes autoimune (tipo 1) pelos extratos *de U. tomentosa e U. guianensis"*. O protocolo experimental foi delineado objetivando-se avaliar a capacidade dos extratos em prevenir a progressão do diabetes através da modulação do perfil imunológico Th1 e Th2, assim como pela estimulação de linfócitos T reguladores.

Artigos

Uncaria tomentosa aqueous-ethanol extract triggers an immunomodulation toward a Th2 cytokine profile

Submitted to Phytotherapy Research (code PTR-10-1001)

Short title: "*Uncaria tomentosa* and immunomodulation"

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Financial support: FAPESP (2008/53113-9)

Abstract

Uncaria tomentosa (Willd.) DC (Rubiaceae) is a large woody vine that is native to the Amazon and Central American rainforests and is widely used in traditional medicine for its immunomodulatory and anti-inflammatory activities. The present work used *in vivo* immunotoxic and *in vitro* immunomodulatory experiments to investigate the effects of a pentacyclic oxindole alkaloid extract from *U. tomentosa* bark on lymphocyte phenotype, Th1/Th2 cytokine production, cellular proliferation and cytotoxicity. For the *in vivo* immunotoxicity testing, BALB/c male mice were treated once a day with 125, 500 or 1250 mg/kg of *U. tomentosa* extract for 28 days. For the *in vitro* protocol, lymphocytes were cultured with 10 to 500 µL/mL of the extract for 48 h. The extract increased the cellularity of splenic white pulp and the thymic medulla and increased the number of T helper lymphocytes and B lymphocytes. Also, a large stimulatory effect on lymphocyte viability was observed. However, mitogen-induced T lymphocyte proliferation was significantly inhibited at higher concentrations of *U. tomentosa* extract. Furthermore, an immunological polarization toward a Th2 cytokine profile was observed. These results suggest that the *U. tomentosa* aqueous-ethanol extract was not immunotoxic to mice and was able to modulate distinct patterns of the immune system in a dose-dependent manner.

Keywords: *Uncaria tomentosa*; immune system; mice; immunotoxicity; cytokines; immunophenotype.

1. Introduction

Uncaria tomentosa (Willd.) DC (Rubiaceae), known as cat's claw, is a large woody vine that is found in the Amazon rainforest and the surrounding tropical areas (Obregón-Vilches, 1997). This species has been shown to demonstrate immunostimulant, cytotoxic, anti-inflammatory and antioxidant activities, and it is commercialized in its natural form or as a phytopharmaceutical derivative (Valente, 2006). Its chemical profile includes the presence of indole and oxindole alkaloids in the whole plant. Other components such as proanthocyanidins, sterols, triterpenoids and carboxyl-alkyl esters have been isolated from the bark (Sandoval et al., 2000; Akesson et al., 2005; Gonçalves et al., 2005; Heitzman et al., 2005). Individually or synergistically, these compounds contribute to the therapeutic properties of the species.

Several extracts from *U. tomentosa* bark with different constituents have exhibited interesting characteristics in terms of immunomodulatory and anti-inflammatory activities. Aqueous extracts with low alkaloid contents have been shown to exhibit anti-inflammatory activities and anti-oxidant, immunostimulant, antitumor, and DNA-repairing properties. Carboxyl-alkyl esters (quinic acid derivatives) and/or polyphenolics may be responsible for these effects (Sandoval et al., 2000; Gonçalves et al., 2005; Akesson et al., 2005). In contrast, a comparative study between the aqueous-ethanol and aqueous extracts from the bark revealed that the former, which contains a high alkaloid content, was more active as an anti-inflammatory agent (Aguilar et al., 2002). Furthermore, a chloroform:methanol 9:1 dilution (v/v) and an acetone extract presented anti-inflammatory activities that were correlated to quinovic acid glycosides and sterols, respectively (Senatore et al., 1989; Aquino et al., 1991).

The effects of these extracts on the transcription factors of cells and their immunological parameters have been described, but the effects vary according to the

experimental conditions. Previous studies have reported that several *U. tomentosa* bark extracts can inhibit TNF-α, iNOS and NF-κB expression and can also increase the synthesis of IL-1β and IL-6, the phagocytosis index and the absolute number of leukocytes (Sandoval et al., 2000; Akesson et al., 2003; Allen-Hall et al., 2010; Lemaire et al., 1999; Wagner et al., 1985; Wurm et al., 1998). This immunomodulatory activity also includes effects on mononuclear cell proliferation. Alkaloid-rich extracts and ethanol extracts from *U. tomentosa* have been shown to inhibit the proliferation of human mononuclear cells that are induced by the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) (Winkler et al., 2004). A study that used a commercial aqueous extract also revealed an inhibition of T and B lymphocyte proliferation without interfering with the production of IL-2 (Akesson et al., 2003). Although the anti-proliferative effects are dominant, the same standardized commercial aqueous extract increased the proliferation of PHA-stimulated murine lymphocytes after *in vivo* treatment (Sheng et al., 2000). These conflicting actions add more complexity to this subject. More recently, Reis et al. (2008) reported that the alkaloid fraction from *U. tomentosa* had an immunomodulatory activity, characterized by a strong inhibition of TNF-α and IFN-γ production by human monocytes that were infected with type-2 Dengue virus.

Previous studies have reported a low incidence of systemic toxicity during *U. tomentosa* treatment. The LD_{50} (median lethal dose) for the aqueous extract was reported to be higher than 8 mg/kg of body weight (Sheng et al., 2000). This study also demonstrated that a commercial aqueous extract did not cause general signs of toxicity; however, the experimental conditions were limited to female Wistar-Furth rats and a low-dose regimen. Further, Wistar rats that were orally treated with 1000 mg/kg of an *U. tomentosa* preparation containing 7.5 mg of total oxindole alkaloids/g presented

lymphocytosis and neutropenia in addition to an increase in kidney weight. However, these results seem to be restricted to an aqueous extract, and the authors did not test for a dose-response relationship (Svendsen and Skydsgaard, 1986 – unpublished data).

Despite the potential immunomodulatory effects of *U. tomentosa*, a lack of research exists regarding its immunotoxic potential. In addition, several controversial immunological effects need to be assessed. The main objective of the present work was to evaluate whether a 28-day oral treatment with an aqueous-ethanol extract from *U. tomentosa* bark would modulate the immune system without triggering a concomitant immunotoxic effect. Additional *in vitro* experiments were used to analyze this extract's effect on lymphocyte proliferation and Th1 and Th2 cytokine profiles.

2. Material and Methods

2.1. Plant material and preparation of the extract

An aqueous-ethanol 1:1 extract that was previously obtained (Valente et al., 2006) from the stem bark of a wild specimen of *U. tomentosa*, collected in Cruzeiro do Sul, Acre, Brazil, and donated by Biosapiens Co., Brazil [voucher data in Miranda et al. (2003)], was used for the assays. The pentacyclic oxindole alkaloid (POA) profile and content of this extract was determined using high-performance liquid chromatography (HPLC) as previously described (Reis et al., 2008 Pereira et al., 2008). Briefly, the crude hydroalcoholic extract was treated with 0.1 N HCl and then partitioned with EtOAc. The resulting aqueous fraction was treated with NH_4OH until a pH of 9 to 10 was reached, and then the fraction was extracted with EtOAc. This alkaloid rich EtOAc fraction was dried and filtered, and the solvent evaporated under low pressure. A MeOH solution of this alkaloid fraction was injected into a reverse-phase HPLC system using a

Lichrocart Lichrospher 5 µm, 125 x 4.6 mm i.d. column and a UV-detector at 245 nm under the same conditions previously described (Laus and Keplinger, 1994). The identification of the POA signals was made by comparing their relative retention times to those of pteropodine. The total alkaloid content was determined using HPLC-UV with the same chromatographic column and a mixture of 45% of MeCN and 55% of an aqueous 30 mM NH_4OAc solution as the mobile phase. The pH of the mobile phase was adjusted to between 6.8 and 7.0, and the analysis was run in isocratic mode with a flow rate of 1.0 mL/min at 60 ºC and detection at 245 nm. Five independent points for the external calibration curve were established using triplicate injections of an isopteropodine solution in MeOH. The curve presented linearity (R^2=0.9846) in the range of 4.75 to 76.0 µg/mL, with a standard deviation of 1.0 µg/mL. The minimum detectable concentration was estimated to be 1.0 µg/mL.

2.2. Animals

Six weeks old pathogen-free BALB/c male mice were purchased from the Multidisciplinary Center of Biological Investigations (CEMIB, Campinas, São Paulo, Brazil) and housed in rooms with controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 55 ± 20%, a 12/12 h light-dark cycle and 4 daily exhaustion periods). All animals received Nuvilab CR-1 commercial food (Nuvital, Brazil) and filtered water *ad libitum* and were submitted to a 2-week acclimation period before the experiment. Experimental protocols were approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, São Paulo, Brazil (protocol number 635).

2.3. Experimental design

BALB/c mice were randomized into groups of 10 animals each. Treatment with *U. tomentosa* extract or its dilution vehicle (alcohol 10%) was given orally for 28 consecutive days. The following groups were used for the *in vivo* evaluations: G1 contained animals treated with the dilution vehicle (alcohol 10%), and G2, G3 and G4 contained animals treated with *U. tomentosa* aqueous-ethanol extract at 125, 500 and 1250 mg/kg of body weight, respectively. Body weights, water and food consumption were evaluated once a week during the experimental period. After 28 days, the animals were euthanized in a CO_2 chamber. Blood and tissue samples were collected for hematological analysis, histopathology and immunophenotyping of splenic lymphocytes.

To evaluate the *in-vitro* immunological effects of the *U. tomentosa* extract, *s*plenocytes were stimulated with the extract (10 to 500 µg/mL) for 48 h. Cellular viability and proliferation were assessed using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The production of Th1 and Th2 cytokines was evaluated using a sensitive cytometric bead array (CBA) in splenocyte culture supernatant.

2.4. Tissue processing and histological analysis

The liver, kidneys, spleen, thymus, mesenteric lymph node and femur were removed and fixed in 10 % buffered formalin (phosphate buffer pH = 7.2) (Carlo Erba Reagents, Italy) for 48 h. The spleen was divided into two portions: one for histology and the other for lymphocyte phenotypic analysis by flow cytometry. The paraffin blocks were cut into 5-µm-thick sections and stained with hematoxylin-eosin (HE)

(Sigma-Aldrich, USA).The histophatological analysis was conduced following previously published criteria by Society of Toxicologic Pathology (Haley et al.,2005).

2.5. Hematological analysis

Blood samples were collected by cardiac puncture and blood smears were immediately prepared on glass slides. The samples were stained by Leishman (Merck Darmstadt, Germany), and the relative counting of granulocytes and mononuclear cells (200 cells/slide) was evaluated using a conventional light microscope at 40x magnification.

2.6. Splenic single cell suspensions

The spleens were pushed against a 50 µm sterile nylon mesh (BD, New Jersey, USA) in Petri dishes containing RPMI-1640 culture medium (Cultilab, Campinas, Brazil). The erythrocytes were lysed using an isotonic solution of ammonium chloride, potassium bicarbonate and ethylenediamine tetraacetic acid (pH 7.2) (Sigma-Aldrich, USA). Cell suspensions were washed twice (200 x g / 10 min), and the pellet was resuspended in RPMI-1640 culture medium that was supplemented with 10% inactivated bovine fetal serum (Cultilab, Campinas, Brazil). Cellular viability was evaluated using trypan blue (Hyclone, Thermo Scientific, USA) and was considered adequate for values greater than 90 %. Cell suspensions were adjusted to 5 x 10^6 cells/mL and cryopreserved in RPM-1640 medium plus 10% dimethyl sulfoxide (Carlo Erba Reagents, Italy) for phenotypic analysis of the lymphocyte subpopulations by flow cytometry.

2.7. Lymphocyte immunophenotyping

Cell aliquots were thawed in a temperature-controlled bath (37 °C) and were immediately transferred to tubes that contained RPMI-1640 medium supplemented with 10% bovine fetal serum. After centrifugation (200 x g/10 min), the pellet was resuspended in 3 mL of an isotonic solution (Advia70, Bayer, Germany) and was washed twice. Cellular viability was evaluated using trypan blue, and these values were considered adequate when they were greater than 90%. The cellular concentration was then adjusted to $1x10^6$ cells/mL, and aliquots (100 µL) were incubated in the dark for 30 min with the following antibodies: FITC rat anti-mouse CD4 (L3T4); PE-Cy5 rat anti-mouse CD8a (Ly-2) and PE rat anti-mouse CD45RA (BD Pharmingen, USA). Splenic lymphocyte subset counts were determined by FACS Calibur analysis (BD Pharmingen, USA) with gating on the total lymphocyte population. Lymphocytes were characterized by their appearance according to Forward Scatter (FC) and Side Scatter (SSC) analyses. For consistency between each flow cytometry analysis, we used the standard calibration bead (BD Pharmingen, USA) to set the forward scatter, side scatter and PMT voltage. For the experimental samples, a corresponding control was used to set gates or positive/negative cell populations.

2.8. Cytotoxicity and proliferation assays

Ten BALB/c mice were used for this experiment, and the single-cell suspensions were obtained as previously described (item 2.6). The cellular concentration was adjusted to $5x10^6$ cells/mL, and each sample was dispensed in triplicate in a 96-well flat-bottom tissue culture plate (Corning Costar, USA) and was treated with the *U. tomentosa* extract at concentrations of 10, 50, 100 and 500 µg/mL. Control samples were treated with 10% ethanol, which was the dilution vehicle. To evaluate lymphocyte

proliferation, the cellular samples were treated with the same concentrations of the extract and were stimulated by mitogen Con A (Sigma-Aldrich, USA) at 10 µg/mL. Control wells lacking Con A were used as a reference for basal levels of lymphocyte proliferation. The plates were incubated for 48 h (37 °C, 5% CO_2). Then, MTT solution (Sigma Aldrich, USA) (5 mg/mL) was added to all of the wells and incubated for 4 h (37 °C, 5% CO_2). The spent media were pipetted out, and DMSO (Carlo Erba reagents, Italy) was added to each well to dissolve the dark blue formazan crystals. The optical density (OD) values were measured using a microtiter plate reader at 570 nm.

2.9. Th1 and Th2 cytokines production

Splenocytes, obtained as previously described (item 2.6), were dispensed in a 48-well flat-bottom tissue culture plate (Corning, Costar, USA) at 5 x 10^6 cells/mL. The samples were stimulated by mitogen Con A (10 µg/mL) and treated with 100 or 500 µg/mL of the extract. The plates were incubated for 48 h (37 °C, 5% CO_2), and the supernantants were collected and stored at -80^o C. Th1 and Th2 cytokines were quantified using a murine Th1/Th2 cytometric bead array (CBA) kit according to the manufacturer's instruction (BD Pharmingen, USA). The FACSCalibur flow cytometer (BD Pharmingen, USA) was calibrated using setup beads, and a total of 3000 events were acquired for each sample. Individual cytokine concentrations (pg/mL) were evaluated by their fluorescence intensities (FL-2 channel) and analyzed based on the standard reference curve of CELLQUEST and CBA software (BD Pharmingen, USA).

2.10. Statistical analysis

The statistical analyses were performed using GraphPad Prism 5.0. The data were tested for normality (Kolmogorov-Smirnov) and homogeneity (Bartlett's), and if

necessary, appropriated transformations were made. Body weights, water and food intake, organ weights, hematology, immunophenotype, cytotoxicity and cytokine results were compared using an analysis of variance (ANOVA). When the results of the ANOVA were significant ($p < 0.05$), the comparison was followed by the Tukey test. For the heterogeneous cases, the Kruskal-Wallis test was applied.

3. Results

3.1. Pentacyclic oxindole alkaloid profile and content

The previous HPLC analysis (Pereira et al., 2008; Reis et al., 2008) of this bioactive aqueous-ethanol extract of *U. tomentosa* indicated the presence of the six pentacyclic oxindole alkaloids that were considered to be chemical markers of the species. The total alkaloid content in the crude extract was calculated as 29.1 mg/g (± 1%), which is in agreement with commercially acceptable plant materials (Figure 1). The total pentacyclic alkaloid pool is usually the marker for assessing the pharmaceutical quality of the cat's claw extracts.

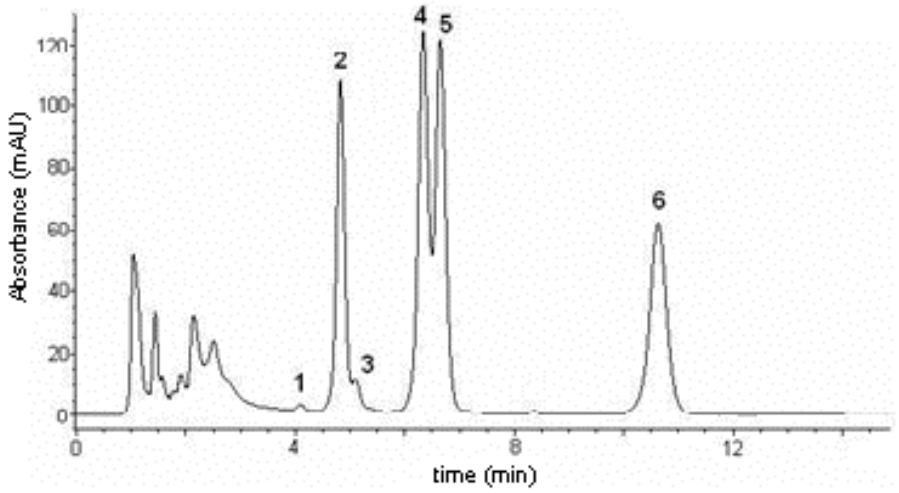

Figure 1 - HPLC-UV pentacyclic oxindole alkaloid profile [Laus and Keplinger (1994) conditions] of the studied Uncaria tomentosa alkaloidal fraction. (1) speciophylline; (2) mitraphylline; (3) uncarine F; (4) pteropodine; (5) isomitraphylline; (6) Isopteropodine. For extract quantification details see Reis et al. (2008).

3.2. General parameters of toxicity and histopathology of the lymphohematopoietic organs, liver and kidneys

No treatment-related mortality of the animals occurred at any tested dose. The clinical analysis did not reveal abnormalities with respect to hair coat, eye color, touch response, strength or salivation. No significant differences were observed for body weight gain and water or food consumption compared to the control group. However, the relative weights of the liver (125 and 500 mg/kg), spleen (125, 500 and 1250 mg/kg) and kidneys (125 mg/kg) were affected by the extract (Table 1).

Table 1 - Body weight[1], body weight gain[2] and organ relative weight[3] in BALB/c male mice treated with U. tomentosa aqueous-ethanol extract during 28 days.

	Control	UT 125 mg/kg	UT 500 mg/kg	UT 1250 mg/kg
Initial body weight	25.9 ± 1.6	25.4 ± 1.2	25.6 ± 1.5	25.7 ± 1.7
Final body weight	30.6 ± 1.5	30.4 ± 0.9	29.7 ± 2.6	29.9 ± 2.7
Body weight gain	4.7 ± 0.8	5.0 ± 1.6	4.2 ± 2.5	4.3 ± 2.6
Liver	4.51 ± 0.34	5.47 ± 0.49**	5.18 ± 0.56*	4.79 ± 0.43
Kidneys	0.79 ± 0.05	0.86 ± 0.07**	0.82 ± 0.06	0.75 ± 0.07
Adrenals	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Spleen	0.30 ± 0.009	0.37 ± 0.05**	0.37 ± 0.02*	0.35 ± 0.05*
Thymus	0.23 ± 0.03	0.21 ± 0.06	0.19 ± 0.04	0.18 ± 0.04

*[1] Body weights are expressed as Mean ± SD; [2] Body weight gain (Mean Final Body weight – Mean Initial Body weight); [3] Organ relative weights (organ weight/final body weight x 100). * and **: Significantly different from control group, $p<0.05$ and $p<0.01$, respectively.*

The spleens from the animals treated with the highest dose of *U. tomentosa* extract (1250 mg/kg) exhibited an increase in marginal zone cellularity and an increase in the incidence of extra-medullary hematopoiesis. Furthermore, the cellularity of the thymic medulla was increased after 1250 mg/kg of the *U. tomentosa* extract. The bone marrow, lymph nodes, kidneys and liver did not exhibit any significant histological alterations.

3.3. Hematological parameters

Compared to the relative values that were observed in the control group, the animals treated with 1250 mg/kg of the *U. tomentosa* extract displayed a significant increase in the relative number of lymphocytes, which was associated with a decrease in granulocytes (Table 2).

Table 2 – Relative counting[1] of mononuclear and polymorphonuclear cells in BALB/c male mice treated with U. tomentosa aqueous-ethanol extract during 28 days.

	Control	UT 125 mg/kg	UT 500 mg/kg	UT 1250 mg/kg
Lymphocytes	82.0 ± 6.9	89.3 ± 5.3	88.9 ± 7.2	91.4 ± 3.7*
Granulocytes	16.1 ± 5.6	7.9 ± 4.6	10.2 ± 6.6	5.5 ± 2.1**
Monocytes	1.90 ± 1.0	2.6 ± 2.4	0.9 ± 1.3	3.3 ± 3.6

[1] *Percentage of mononuclear and polymorphonuclear cells, expressed as Mean ± SD (200 cells/animal were counted). * and **: Significantly different from control group, p<0.05 and p<0.01, respectively.*

3.4. Phenotypic analysis of splenic lymphocyte subpopulations

A higher percentage of mature $CD4^+$ T lymphocytes was observed in the groups that were treated with 125 mg/kg and 500 mg/kg of *U. tomentosa*. These same doses caused a non-significant trend toward an increased percentage of immature $CD4^+$ $CD8^+$ T cells. In contrast, an increased percentage of $CD45RA^+$ B lymphocytes was present in the group that was treated with the highest dose of the extract (1250 mg/kg) when compared to the control and the 125 and 500 mg/kg treated groups (Table 3).

Table 3 – Phenotypic analysis[1] of splenic lymphocyte subsets in BALB/c male mice treated with U. tomentosa aqueous-ethanol extract during 28 days.

	Control	UT 125 mg/kg	UT 500 mg/kg	UT 1250 mg/kg
$CD4^+$ T lymphocytes	21.3 ± 0.3	27.8 ± 3.4*	32.3 ± 3.0*	13.35 ± 1.57*
$CD8^+$ T lymphocytes	12.2 ± 1.3	14.3 ± 2.7	13.6 ± 1.4	7.00 ± 1.9*
$CD4^+CD8^+$ T lymphocytes	1.8 ± 0.5	3.14 ± 1.5	2.42 ± 1.7	1.16 ± 0.3
$CD45RA^+$ B lymphocytes	63.5 ± 1.6	54.5 ± 3.9	51.76 ± 0.8	80.1 ± 4.3*

[1] *Values expressed as percentage of lymphocyte subsets (Mean ± SD). 10.000 cells/animal were counted. *: Significantly different from control group, p<0.05.*

3.5. Effect of Uncaria tomentosa on lymphocyte viability and proliferation

The *U. tomentosa* aqueous-ethanol extract did not cause any toxicity that affected the lymphocyte's survival. In fact, all of the tested concentrations resulted in increased cellular viability compared to the values that were observed in the untreated control group (Figure 2). Nevertheless, higher concentrations of the extract (50 to 500 µg/mL) caused an inhibition in T-lymphocyte proliferation after mitogenic stimulus, resulting in proliferation indexes that were lower than those observed in the untreated control samples (Figure 3).

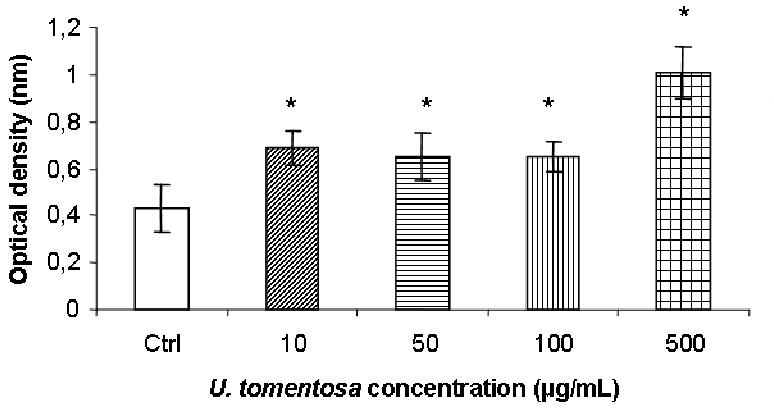

*Figure 2 – Evaluation of splenic lymphocyte viability by the MTT colorimetric assay after treatment with U. tomentosa aqueous-ethanol extract (10-500 µg/mL). Values are expressed as optical density (Mean ± SD). * : Significantly different from control, p<0.001.*

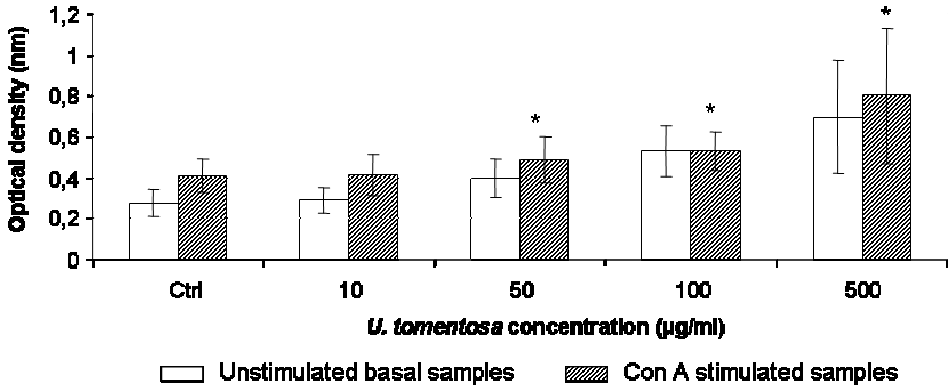

*Figure 3 – Proliferation of splenic lymphocytes treated with U. tomentosa aqueous-ethanol extract. Values are expressed as optical density (Mean ± SD). Proliferation index was calculated following the formula: OD from Con A samples/ OD from basal samples. * indicates inhibition of the mitogen-induced lymphocyte proliferation, compared to the untreated control samples, $p<0.05$.*

3.6. Th1 and Th2 cytokine production

A standard curve with cytokine concentrations ranging from 0 to 5000 pg/mL was constructed for this assay, revealing a fitting-accuracy of 99.9% for each tested cytokine (data not shown). The *U. tomentosa* aqueous-ethanol extract increased the mitogen-induced production of IL-4 and IL-5 and caused a strong inhibition of IFN-γ at the highest tested concentration (500 µg/mL). In addition, both concentrations, 100 and 500 µg/mL, inhibited the production of IL-2. Despite the absence of changes in the TNF-α level at 500 µg/mL, the lowest tested concentration (100 µg/mL) led to a significant increase in the concentration of this pro-inflammatory cytokine by murine splenocytes (Figure 4).

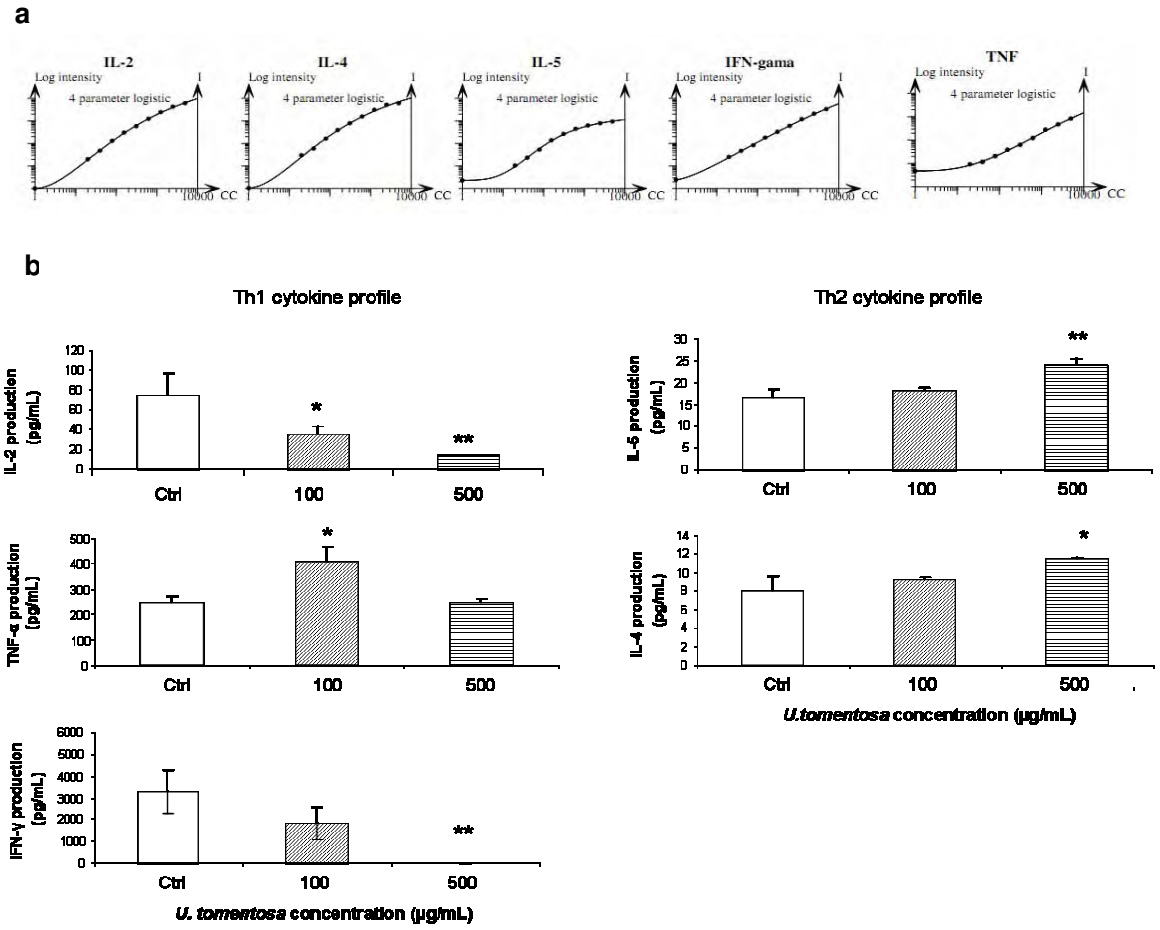

*Figure 4 – Phenotipic analysis of splenic lymphocyte subsets effect of U. tomentosa on Th1 and Th2 cytokine production. a) Standard curves with different cytokine concentrations (0-5000 pg/mL), revealing a fitting accuracy of 99.9%. b) Mitogen-induced Th1 and Th2 cytokine production by murine splenocytes after in-vitro treatment with 100 and 500 µg/mL of U. tomentosa aqueous-ethanol extract. Values are expressed as Mean ± SD (pg/mL). * and ** : Significantly different from control samples, p<0.01 and p<0.001, respectively.*

4. Discussion

Uncaria tomentosa is a medicinal plant that possesses immunomodulatory and anti-inflammatory properties. Previous studies correlated six pentacyclic oxindole alkaloids (POA) that are present in its leaves, bark and roots to the immunomodulatory property of this species (Winkler et al., 2004). As a result of this finding, several

phytopharceuticals that were based on the bark ethanol extract and the alkaloid-rich fraction from *U. tomentosa* have been standardized on the basis of their oxindole alkaloid content (Keplinger et al., 1989; 1994). However, studies concerning the immunotoxicity and the immunomodulatory potential of such compounds still need to be conducted. As a result, this immunomodulating and antiviral *U. tomentosa* bark extract (aqueous-ethanol 1:1), which has been shown to be effective for Dengue fever (Reis et al., 2008), was investigated in the present study to determine its immunotoxic potential. Our results demonstrated that this extract has a low potential for systemic toxicity. These findings are consistent with previous work that demonstrated that an LD50 for an aqueous extract was higher than 8 g/kg (Sheng et al., 2000). Alterations such as an increase in the relative weights of the liver, spleen and kidneys were observed; however, no treatment-related mortality was observed. Furthermore, there were no signs of structural changes or of an enzymatic induction that suggested a toxic effect. Additionally, the higher splenic relative weight could be attributed, at least partially, to the increased incidence of extra-medullary hematopoiesis and to the enlargement of the marginal zone. These findings are relevant because these changes have not been described in previous toxicity studies with *U. tomentosa* extracts and there is a lack of reports that determine a dose-response relationship for this aqueous-ethanol extract following repeated dosing.

Concerning the immunomodulatory potential of this extract, treatment with different doses of *U. tomentosa* resulted in an increase in the relative number of lymphocytes, in a similar manner to the aqueous extract effects (Sheng et al., 2000). In addition, using a flow cytometry analysis, we revealed that this extract increased the number of lymphocytes carrying the CD4 (125 and 500 mg/kg) and CD45RA molecules (1250 mg/kg). This result suggests that this extract has the potential to modulate distinct

patterns of the immune system in a dose-dependent manner. $CD4^+$ T cells are viewed as the major orchestrators of the immune system, which can be differentiated into distinct lineages such as Th1 and Th2 cells, depending upon their microenvironment (Zhu and Paul, 2010). Based on the *in vitro* data, a concentration-dependent immunostimulation was achieved, as observed by the increased number of viable lymphocytes, particularly at the highest extract concentration (500 µg/mL). Because *U. tomentosa* reduced the *in vitro* proliferation of lymphocytes that were stimulated by the T-cell mitogen Con A, the increased cellular viability may be related mainly to $CD45RA^+$ B cells. This result is in accordance with the *in vivo* increase of $CD45RA^+$ B lymphocytes at the highest dose (1250 mg/kg) and with the splenic histology, which revealed an enlargement of the splenic marginal zone, a region that is populated mainly by B cells. In addition, our results suggested that an alternative mechanism through which the same dose of *U. tomentosa* that was able to increase $CD4^+$ cells also promoted a trend toward an increased number of $CD4^+CD8^+$ T lymphocytes. These double-positive lymphocytes are considered to be partially immunocompetent cells that undergo post thymic maturation at the periphery, becoming functionally mature $CD4^+$ T cells (Jiménez et al., 2002). Therefore, *U. tomentosa* may stimulate the transition of $CD4^+CD8^+$ lymphocytes to a mature $CD4^+$ helper phenotype. This possibility is partially supported by a clear increase in the cellularity of thymic medulla, as determined by a careful histological analysis. Literature reports have suggested that many of the immune-related effects of medicinal plants could be due to cytokine modulation (Spelman et al., 2006). The dose-dependent increase in the number of $CD4^+$ T and $CD45RA^+$ B cells could suggest a Th2 polarization, i.e., this extract is determining a differentiation of Th0 cells toward Th2 cells. Our findings reinforce this possibility by showing that splenic cells stimulated *in vitro* with Con A and the highest extract concentration (500 µg/mL) produced

significant levels of the Th2 cytokines IL-4 and IL-5. Under these same conditions, IL-2 and IFN-γ production, which are considered Th1 type cytokines, were clearly inhibited. This finding is in agreement with the classical literature that reports that IL-4 promotes the differentiation of Th2 cells *in vitro* through its action on the transcription factor STAT6 and the upregulation of GATA-binding protein (GATA3), which is the master regulator of Th2 differentiation (Zheng and Flavell, 1997). To ensure that these results were not related to alterations in cell viability, MTT assays were performed to measure cell proliferation, confirming that the extract was not cytotoxic to splenic lymphocytes at any of the tested concentrations.

From these findings, it was concluded that the aqueous-ethanol extract from *U. tomentosa* bark did not cause significant systemic toxicity or immunotoxicity. This extract was endowed with a clear immunomodulatory activity that affected lymphocyte subsets and promoted a cytokine polarization toward a Th2 profile, suggesting its potential to treat Th1 immunomediated disorders.

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Toxicity and immunological effects of the ethanol extract from leaves of *Uncaria guianensis*

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Abstract

Uncaria guianensis. (Rubiaceae) is a large woody vine that is native to the Amazon and Central American rainforests and widely used in traditional medicine for its immunomodulatory and anti-inflammatory activities. The present work used *in vivo* immunotoxic and *in vitro* immunomodulatory experiments to investigate the effects of an ethanol extract from *U. guianensis* leaves on lymphocyte phenotype, Th1/Th2 cytokine production, cellular proliferation and cytotoxicity. For the *in vivo* immunotoxicity testing, BALB/c male mice were orally treated once a day with 125, 500 or 1250 mg/kg of *U. guianensis* extract for 28 days. For the *in vitro* protocol, lymphocytes were cultured with 10 to 500 μL/mL of the extract for 48 h. The extract caused a significant reduction in body weight gain, as well as in thymus and kidney relative weights. Spleens from animals treated with the extract presented higher relative weights, larger marginal zones and more evident germinal centers. Moreover, increased percentage of splenic $CD4^+$ T cells and peripheral monocytosis were observed *in vivo*. The *in vitro* immunomodulatory effect of the extract was demonstrated by the inhibition of mitogen-induced production of Th1 cytokines and IL-5, reinforcing its potential to treat inflammatory and allergic disorders.

Keywords: *Uncaria guianensis*; immune system; mice; immunotoxicity; cytokines; immunophenotype.

1. Introduction

The species *Uncaria guianensis* and *U. tomentosa,* known as cat's claw, are large woody vines native to the Amazon rainforest and other tropical areas of South and Central America (Ridsdale, 1978). They have been used in traditional medicine to treat several ills such as gastritis, gastric ulcers, cancer, arthritis, asthma and inflammatory conditions (Jones, 1995; Obregón-Vilches, 1997; Revilla, 2002). However, in spite of several studies reporting the pharmacological and toxicological properties of *U. tomentosa* (Heitzman et al., 2005), few works have explored these subjects in *U. guianensis*. Sandoval et al. (2002) reported that an aqueous extract from the barks of *U. guianensis* displays the capacity to scavenge free-radicals, to quench lipid peroxidation and also to inhibit the mitogen-induced production of the pro-inflammatory cytokine TNF-α *in vitro.* Another study conduced by Carvalho et al. (2006) demonstrated effective anti-inflammatory and anti-allergic activities, both *in vivo and in vitro*. In this study, the ethanolic extract from leaves of *U. guianensis* reduced murine paw edema and pleural exudation, whereas inhibited mitogen-induced IL-5, nitric oxide and CXCL8 production by peritoneal macrophages and splenocytes. Clinical trials have focused on the anti-inflammatory properties of the species. Piscoya et al. (2001) described that an aqueous extract from the bark of *U. guianensis* was an effective therapy for patients with osteoarthritis of knee through the modulation of NF-κB pathway and TNF-α production. Moreover, a commercial extract containing an association of *U. guianensis* and *Lepidium meyenii* demonstrated potent chrondoprotective effect through the stimulation of insulin-like growth factor 1 (IGF-1) and by preventing the catabolic action of IL-1ß (Miller, 2006). Furthermore, an ethanolic extract from *U. guianensis* displayed an effective antimicrobial activity against a multi-drug resistant strain of *Staphylococcus aureus* (Correia et al., 2008).

Thus, in order to analyze the toxicity and immunological effects of a bioactive ethanol extract from *U. guianensis* leaves (Carvalho et al, 2006), a 28-day oral treatment was employed to evaluate whether this extract could modulate the immune system without triggering a concomitant immunotoxic effect. Additional *in vitro* experiments were used to analyze its effect on lymphocyte proliferation and Th1 and Th2 cytokine profiles.

2. Materials and Methods

2.1. Plant material, extraction and quantification of alkaloids

The *U. guianensis* specimen used in this work were collected in Juruena, Mato Grosso state (12°50' S, 58°55' W; 277 m elevation) in the Brazilian Amazon rainforest, and was identified by the botanist Pierro Delprete (New York Botanic Garden). A voucher was deposited in the Central Herbarium of the Universidade Federal do Mato Grosso, Brazil, under No. 24715. The ethanol extract from *U. guianensis* leaves was obtained by exhaustive extraction at room temperature, as previously described. Its pentacyclic oxindole alkaloid (POA) profile and content were achieved by HPLC-UV using an alkaloid enriched fraction obtained by acid-base partition of the ethanol extract. Thus, 100.4, 453.9 and 520.6 mg of the crude alcoholic extract were ultrasonically (15 min) solubilized in parallel with HCl 0.1N (0.15 mL/mg of the extract), and then partitioned with EtOAc (same volume of the HCl solution, three times). The resulting aqueous fractions were treated with NH_4OH until pH 9-10 and extracted with EtOAc (Valente et al., 2006). These alkaloid rich EtOAc fractions were then dried with anhydrous Na_2SO_4 and the solvent evaporated under low pressure at 37°C yielding 1.8, 17.9 and 12.2 mg (2.6 ± 1.1 %) of the dried alkaloid fractions,

respectively. Solutions of 72.0, 89.5 and 61.0 µg/mL, respectively, were then made in MeOH and filtered through 0.45 µm nylon membranes and injected (10 µl) into the HPLC system. Elution was carried out with a mixture of 45% of MeCN/55% of aqueous 30 mM NH_4OAc solution, pH adjusted at 6.8-7.0, isocratic mode, flow rate of 1.0 mL/min, at 60ºC and detection at 245 nm. The alkaloids peaks were identified by retention time relative to isopteropodine (Pereira et al., 2008). POA samples were quantified by external standardization relative to mitraphylline.

2.2. Animals

Four weeks old, pathogen-free BALB/c male mice were purchased from the Multidisciplinary Center of Biological Investigations (CEMIB, Campinas, São Paulo, Brazil) and housed in rooms with controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 55 ± 20%, a 12/12 h light-dark cycle and 4 daily exhaustion periods). All animals received Nuvilab CR-1 commercial food (Nuvital, Brazil) and filtered water *ad libitum* and were submitted to a 2-week acclimation period before the experiment. Experimental protocols were approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, São Paulo, Brazil (protocol number 635).

2.3. Experimental design

BALB/c mice were randomized into groups of ten. Treatment with *U. guianensis* extract or its dilution vehicle (alcohol 10%) was given orally for 28 consecutive days. The following groups were used for the *in vivo* evaluations: G1 contained animals treated with the dilution vehicle and G2, G3 and G4 contained animals treated with *U. guianensis* ethanol extract at 125, 500 and 1250 mg/kg of body weight, respectively.

Body weights, water consumption and food consumption were evaluated once a week during the experimental period. After 28 days, the animals were euthanized in a CO_2 chamber. Blood and tissue samples were collected for hematological analysis, histopathology and immunophenotyping of splenic lymphocytes.

To evaluate the *in-vitro* immunological effects of the *U. guianensis*, *s*plenocytes were stimulated with the extract (10 to 500 µg/mL) for 48 h. Cellular viability and proliferation were assessed using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The production of Th1 and Th2 cytokines was evaluated using a sensitive cytometric bead array (CBA) in splenocyte culture supernatant.

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2.4. Tissue processing and histological analysis

The liver, kidneys, spleen, thymus, mesenteric lymph node and femur were removed and fixed in 10 % buffered formalin (phosphate buffer pH = 7.2) (Carlo Erba Reagents, Italy) for 48 h. The spleen was divided into two portions: one for histology and the other for lymphocyte phenotypic analysis by flow cytometry. The paraffin blocks were cut into 5-µm-thick sections and stained with hematoxylin-eosin (HE) (Sigma-Aldrich, USA).The histophatological analysis was conduced following previously published criteria by Society of Toxicologic Pathology (Haley et al.,2005).

2.5. Hematological analysis

Blood samples were collected by cardiac puncture and blood smears were immediately prepared on glass slides. The samples were stained by Leishman (Merck Darmstadt, Germany), and the relative counting of granulocytes and mononuclear cells

(200 cells/slide) was evaluated using a conventional light microscope at 40x magnification.

2.6. Splenic single cell suspensions

The spleens were pushed against a 50 µm sterile nylon mesh (BD, New Jersey, USA) in Petri dishes containing RPMI-1640 culture medium (Cultilab, Campinas, Brazil). The erythrocytes were lysed using an isotonic solution of ammonium chloride, potassium bicarbonate and ethylenediamine tetraacetic acid (pH 7.2) (Sigma-Aldrich, USA). Cell suspensions were washed twice (200 x g / 10 min), and the pellet was resuspended in RPMI-1640 culture medium that was supplemented with 10% inactivated bovine fetal serum (Cultilab, Campinas, Brazil). Cellular viability was evaluated using trypan blue (Hyclone, Thermo Scientific, USA) and was considered adequate for values greater than 90 %. Cell suspensions were adjusted to 5 x 10^6 cells/mL and cryopreserved in RPM-1640 medium plus 10% dimethyl sulfoxide (Carlo Erba Reagents, Italy) for phenotypic analysis of the lymphocyte subpopulations by flow cytometry.

2.7. Lymphocyte immunophenotyping

Cell aliquots were thawed in a temperature-controlled bath (37 °C) and were immediately transferred to tubes that contained RPMI-1640 medium supplemented with 10% bovine fetal serum. After centrifugation (200 x g/10 min), the pellet was resuspended in 3 mL of an isotonic solution (Advia70, Bayer, Germany) and was washed twice. Cellular viability was evaluated using trypan blue, and these values were considered adequate when they were greater than 90%. The cellular concentration was then adjusted to 1x10^6 cells/mL, and aliquots (100 µL) were incubated in the dark for 30

min with the following antibodies: FITC rat anti-mouse CD4 (L3T4); PE-Cy5 rat anti-mouse CD8a (Ly-2) and PE rat anti-mouse CD45RA (BD Pharmingen, USA). Splenic lymphocyte subset counts were determined by FACS Calibur analysis (BD Pharmingen, USA) with gating on the total lymphocyte population. Lymphocytes were characterized by their appearance according to Forward Scatter (FC) and Side Scatter (SSC) analyses. For consistency between each flow cytometry analysis, we used the standard calibration bead (BD Pharmingen, USA) to set the forward scatter, side scatter and PMT voltage. For the experimental samples, a corresponding control was used to set gates or positive/negative cell populations.

2.8. Cytotoxicity and proliferation assay

Ten BALB/c mice were used for this experiment, and the single-cell suspensions were obtained as described at the item 2.6. The cellular concentration was adjusted to $5x10^6$ cells/mL, and each sample was dispensed in triplicate in a 96-well flat-bottom tissue culture plate (Corning Costar, USA) and was treated with the *U. guianensis* extract at concentrations of 10, 50, 100 and 500 µg/mL. Control samples were treated with 10% ethanol, which was the dilution vehicle. To evaluate lymphocyte proliferation, the cellular samples were treated with the same concentrations of the extract and were stimulated by the mitogen Con A (Sigma-Aldrich, USA) at 10 µg/mL. Control wells lacking Con A were used as a reference for basal levels of lymphocyte proliferation. The plates were incubated for 48 h (37 °C, 5% CO_2). Then, MTT solution (Sigma Aldrich, USA) (5 mg/mL) was added to all of the wells and incubated for 4 h (37 °C, 5% CO_2). The spent media were pipetted out, and DMSO (Carlo Erba reagents, Italy) was added to each well to dissolve the dark blue formazan crystals. The optical density (OD) values were measured using a microtiter plate reader at 570 nm.

2.9. Th1 and Th2 cytokines production

Splenocytes, obtained as described at the item 2.6, were dispensed in a 48-well flat-bottom tissue culture plate (Corning, Costar, USA) at 5 x 10^6 cells/mL. The samples were stimulated by mitogen Con A (10 µg/mL) and treated with 100 or 500 µg/mL of the extract. The plates were incubated for 48 h (37 °C, 5% CO_2), and the supernantants were collected and stored at -80° C. Th1 and Th2 cytokines were quantified using a murine Th1/Th2 cytometric bead array (CBA) kit according to the manufacturer instructions (BD Pharmingen, USA). The FACSCalibur flow cytometer (BD Pharmingen, USA) was calibrated using setup beads, and a total of 3000 events were acquired for each sample. Individual cytokine concentrations (pg/mL) were evaluated by their fluorescence intensities (FL-2 channel) and analyzed based on the standard reference curve of CELLQUEST and CBA software (BD Pharmingen, USA).

2.10. Statistical analysis

The statistical analyses were performed using GraphPad Prism 5.0. The data were tested for normality (Kolmogorov-Smirnov) and homogeneity (Bartlett's), and if necessary, appropriated transformations were made. Body weights, water and food intake, organ weights, hematology, immunophenotype, cytotoxicity and cytokine results were compared using an analysis of variance (ANOVA). When the results of the ANOVA were significant ($p < 0.05$), the comparison was followed by the Tukey test. For the heterogeneous cases, the Kruskal-Wallis test was applied.

3. Results

3.1. Chemical analysis of the Uncaria guianensis extract

The HPLC analysis of the alkaloid fraction of ethanol extract of the leaves of *U. guianensis* showed five of the pentacyclic oxindole alkaloids considered as the chemical marker of the species *U. tomentosa* (Laus and Keplinger, 1994). Total alkaloid content in the crude extract was calculated as 6.5 mg/g (rel. s.d. 4.5%). This Brazilian specimen presented in its leaves, low concentration of speciophylline (1) with mitraphylline (2), pteropodine (4), isomitraphylline (5) and isopteropodine (6) as major compounds (Figure 1). In addition, from this extract have been previously isolated the flavonol glycoside kaempferol-3,7-O-(□)-dirhamnoside (kaempferitrin) (**7**) (Figure 2) and the HPLC-DAD-MS flavonoid profile of this extract showed that this compound was the major polyphenolic component (Valente et al., 2009) (Figure 3).

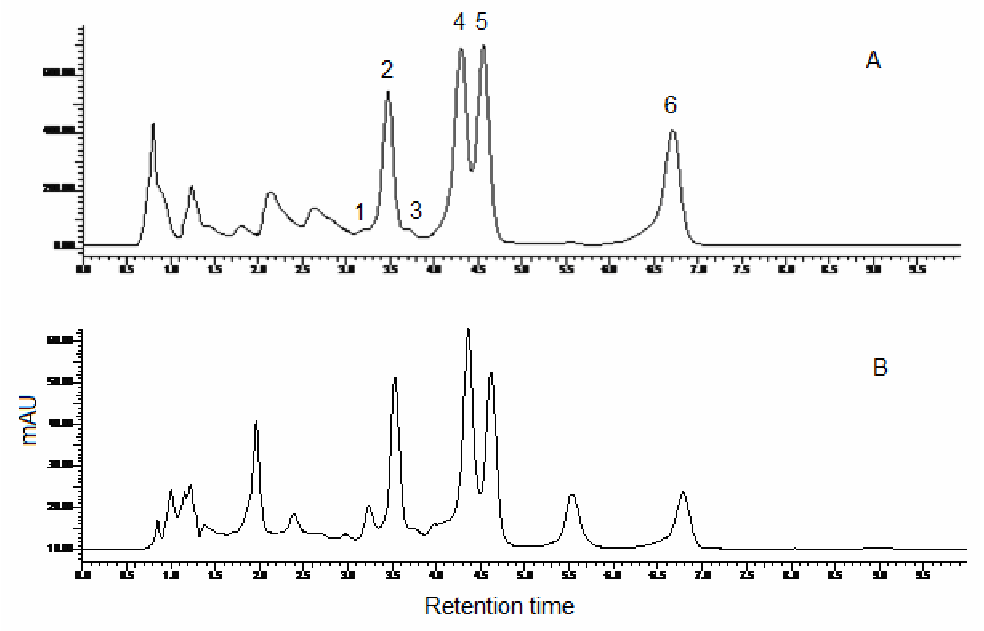


Figure 1 - A: HPLC-UV oxindole alkaloid profile of the reference sample (Uncaria tomentosa); B: HPLC-UV oxindole profile of the ethanol extract of the leaves of Uncaria guianensis

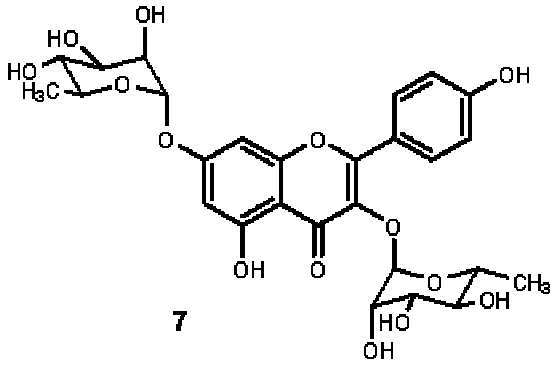


Figure 2 – Structure of the flavonol glycoside kaempferol-3,7-O-(a)-dirhamnoside (kaempferitrin)

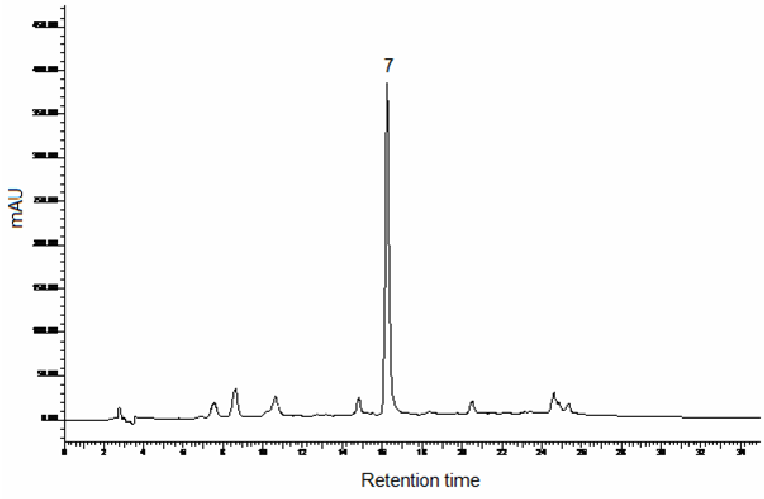


Figure 3 – HPLC-UV flavonoid profile of the ethanol extract of the leaves of Uncaria guianensis.

3.2. General parameters of toxicity and histopathological analysis of liver, kidneys and lymphohematopoietic organs

No treatment-related mortality occurred at any tested dose. The clinical analysis did not reveal abnormalities with respect to hair coat, eye color, touch response, strength or salivation. No significant differences were observed regarding food and water intake, however the mean body weight gain was reduced in the animals treated with the three

tested doses of *U. guianensis* (125, 500 and 1250 mg/kg) ($0.001 < p < 0.05$). Furthermore, organ relative weight was reduced in kidneys (125, 500 and 1250 mg/kg) and thymus (1250 mg/kg) ($p<0.05$) and increased in spleen at 125 mg/kg ($p<0.01$) (Table 1).

Table I – Body weight[1], body weight gain[2] and organ relative weight[3] in BALB/c male mice treated with an ethanolic extract from *U. guimensis* during 28 consecutive days

	Control	UG 125 mg/kg	UG 500 mg/kg	UG 1250 mg/kg
Inital body weight	25.9 ± 1.6	28.14 ± 2.26	26.85 ± 2.26	27.57 ± 2.57
Final body weight	30.6 ± 1.5	28.57 ± 2.70	29.14 ± 2.85	29.28 ± 2.13
Body weight gain	4.70 ± 0.8	0.42 ± 2.22***	2.29 ± 0.90*	1.71 ± 2.13**
Liver	4.51 ± 0.34	5.15 ± 0.74	4.79 ± 0.70	4.76 ± 0.52
Kidneys	0.79 ± 0.05	0.74 ± 0.2*	0.72 ± 0.06*	0.69 ± 0.07*
Spleen	0.30 ± 0.009	0.53 ± 0.41**	0.33 ± 0.06	0.33 ± 0.06
Thymus	0.23 ± 0.03	0.20 ± 0.08	0.21 ± 0.05	0.17 ± 0.03*

*[1] Body weights are expressed as Mean ± SD. [2] Body weight gain (Mean final body weight – Mean initial body weight). [3] Relative organ weight (organ weight/final body weight x 100). *, ** and *** significantly different from control group; p<0.05, p<0.01 and p<0.001, respectively.*

Spleens from animals treated with 125, 500 and 1250 mg/kg of the extract exhibited an increase in marginal zone cellularity and more evident germinal centers. This observation was even more significant for the lowest dose (125 mg/kg). Curiously, one animal treated with *U. guianensis* at 125 mg/kg developed an intense mononuclear infiltration around the peri-vascular areas of liver and dispersed within the kidneys. Also, the structure of spleen, thymus and lymph nodes were severely affected, exhibiting an expansion of splenic white pulp and thymic medulla, increased extra-medullary hematopoiesis and intense incidence of apoptosis. The immunohistochemical

analysis was negative for B lymphocytes ($CD20^+$), but positive for $CD3^+$ T lymphocytes, Tdt (terminal deoxynucletidyl transferase, expressed in malignant tumor of lymphoblastic lineage) and Ki67 (cellular marker for cellular proliferation), indicating a diagnosis of T cell acute lymphoblastic lymphoma.

3.3. Hematological analysis

Compared to the standard values observed in the control group, the relative number of monocytes was higher in the group treated with 1250 mg/kg of *U. guianensis* ($p<0.001$). The counting of lymphocytes, granulocytes, eosinophils and basophils did not differ among the groups (Table 2).

Table 2 – Relative counting[1] of mononuclear and polymorphonuclear cells in BALB/c male mice treated with an ethanolic extract from *U. guianensis* during 28 consecutive days.

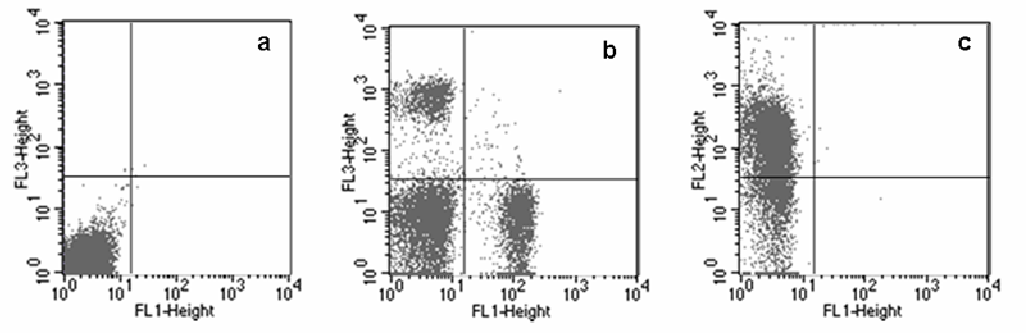
	Control	UG 125 mg/kg	UG 500 mg/kg	UG 1250 mg/kg
Lymphocytes	82.0 ± 6.9	77.7 ± 7.1	79.0 ± 5.0	73.0 ± 7.5
Granulocytes	16.1 + 5.6	18.5 ± 6.6	18.0 ± 5.1	14.0 ± 4.9
Monocytes	1.90 ± 1.0	3.7 ⊥ 1.7	3.0 ⊥ 1.2	13.0 ± 6.4*

[1] *Percentage of mononuclear and polymorphonuclear cells, expressed as Mean ± SD (200 cells/animals were counted). * significantly different from control group; p<0.001.*

3.4. Phenotipic analysis of splenic lymphocyte subpopulations

The phenotypic analysis of lymphocyte subsets indicated an increase in the percentage of mature $CD4^+$ T lymphocytes in the groups treated with 500 and 1250 mg/kg of *U. guianensis* ($0.001<p<0.05$), followed by a decrease in the percentage of

immature $CD4^+CD8^+$ T lymphocytes in all tested doses (125, 500 and 1250 mg/kg) ($p<0.05$). The number of $CD45RA^+$ B cells was not affected by the extract (Figure 4).



	Control	UG 125 mg/kg	UG 500 mg/kg	UG 1250 mg/kg
$CD4^+$ T lymphocytes	21.3 ± 0.3	22.7 ± 0.9	23.0 ± 1.0*	24.0 ± 1.5**
$CD8^+$ T lymphocytes	14.3 ± 2.7	13.0 ± 1.4	13.5 ± 1.3	13.6 ± 1.1
$CD4^+CD8^+$ T lymphocytes	1.8 = 0.5	1.0 ÷ 0.2*	1.0 + 0.1*	1.0 ± 0.4*
$CD45RA^+$ B lymphocytes	63.5 ± 1.4	63.8 ± 3.3	61.0 ± 1.3	61.1 ± 3.4

*Figure 4 – Phenotipic analysis of splenic lymphocyte subsets. a, b and c: Dot plots representing animals treated with the highest dose of U. guianensis (1250 mg/kg). a: negative fluorescence control; b: FL1 FITC $CD4^+$ and FL2 PE $CD8^+$, c: FL2 PE $CD45RA^+$. Values are expressed as percentage of lymphocyte subsets (Mean ± SD), 10000 cells/animals were counted. * and ** Significantly different from control, $p<0.05$ and $p<0.001$, respectively.*

3.5. Effect of U. guianensis on lymphocyte viability and proliferation

U. guianensis ethanolic extract did not affect lymphocyte survival. In fact, the concentrations of 10 and 50 µg/mL were responsible for an increase in lymphocyte viability relative to the untreated control samples ($p<0.05$) (Figure 5). However, higher concentrations of *U. guianensis* (100 and 500 µg/mL) caused an inhibition in T-

lymphocyte proliferation after mitogenic stimulus, resulting in proliferation indexes that were lower than those observed in the untreated control samples ($p<0.05$) (Figure 6).

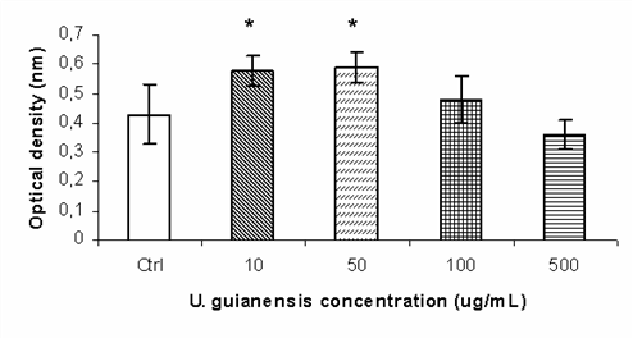


*Figure 5 – Evaluation of splenic lymphocyte viability by the MTT colorimetric assay after treatment with U. guianensis ethanolic extract in vitro (10-500 µg/mL). Values are expressed as optical density (Mean ± SD), * Significantly different from control, p<0.05.*

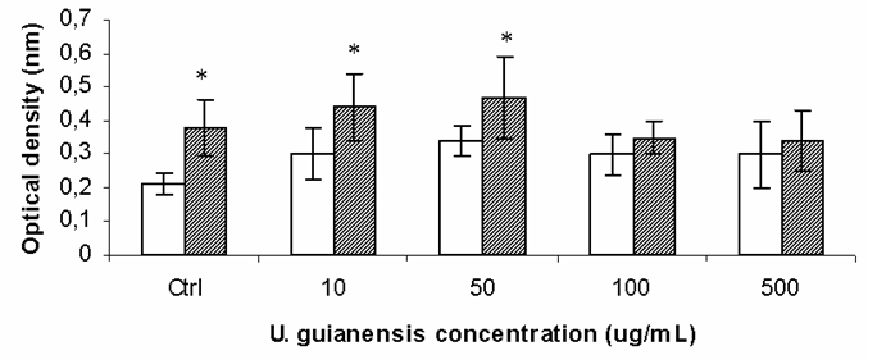


*Figure 6 - Proliferation of splenic lymphocytes treated with U. guianensis ethanolic extract in vitro (10-500 µg/mL) and stimulated by the mitogen Con A. Values are expressed as optical density (Mean ± SD). * indicates increased lymphocyte proliferation in samples stimulated by Con A, compared to their respective basal references, p<0.001.*

3.6. Production of Th1 and Th2 cytokines

A standard curve with cytokine concentrations ranging from 0 to 5000 pg/mL was constructed for this assay, revealing a fitting-accuracy of 99.9% for each tested

cytokine (data not shown). The *U. guianensis* ethanolic extract significantly inhibited the mitogen-induced production of the cytokines INF-γ, TNF-α, IL-2 and IL-5, particularly at 500 µg/mL ($0.01<p<0.05$). However, the extract did not affect the production of IL-4 at any tested concentration ($p>0.05$) (Figure 7).

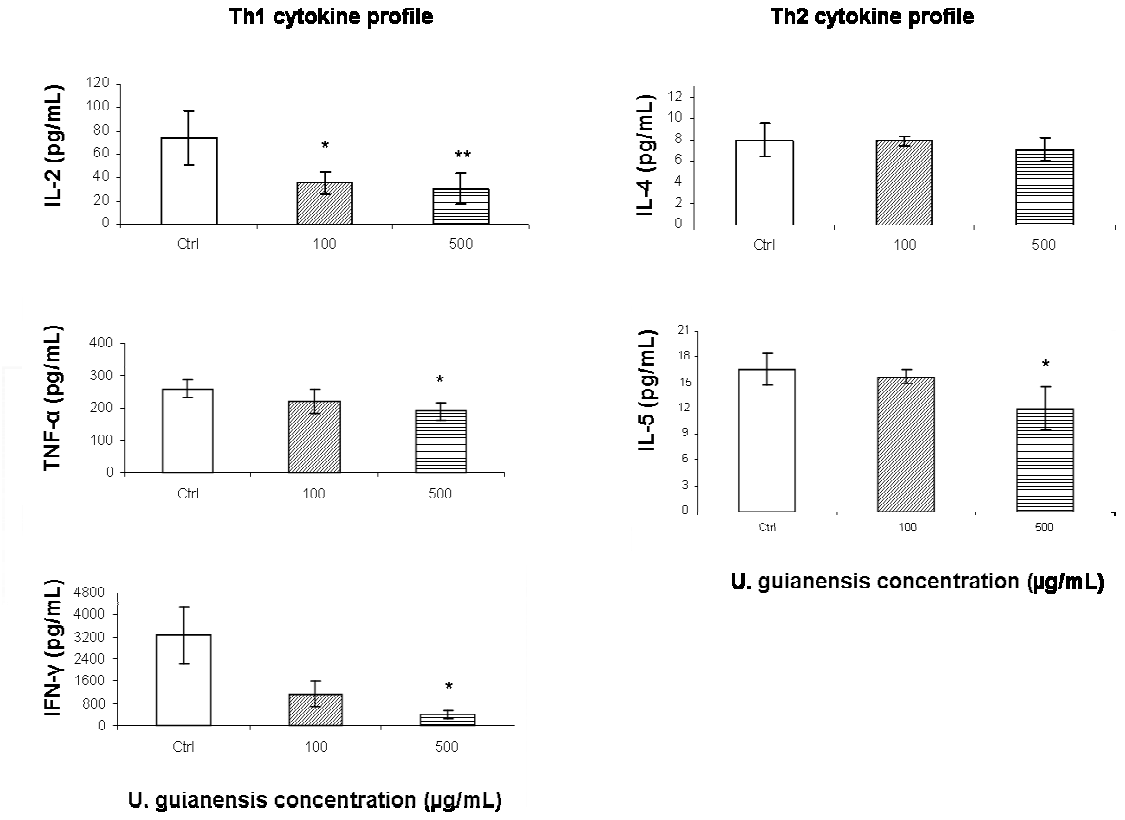


*Figure 7 – Mitogen-induced Th1 and Th2 cytokine prodruction by murine splenocytes after in-vitro treatment with 100 and 500 µg/mL of U. guianensis ethanolic extract. Values are expressed Mean ± SD (pg/mL). . * and ** Significantly different from control samples, $p<0.05$ and $p<0.001$, respectively.*

4. Discussion

Even tough *U. guianensis* has been widely used in traditional medicine; studies on its toxicity still need to be conduced. As a result, an authenticated ethanol extract from the leaves of *U. guianensis* was hereafter investigated to characterize both, the general signs of toxicity, including immunotoxicity, and the imunomodulatory potential of the species.

Previous chemical studies of *U. guianensis* revealed indole and oxindole alkaloids (whole plant) (Carbonezi et al., 2004; Laus and Keplinger, 1994), proanthocyanidins (bark) (Sandoval et al., 2002), flavonols (leaf and bark) (Alvarez et al., 1988; Valente et al., 2009) and triterpenoid glycosides (bark) (Alvarez et al., 1988; Yépez et al., 1991).

In this study, five pentacyclic oxindole alkaloids (speciophylline, mitraphylline, pteropodine, isomitraphylline and isopteropodine) and other non-identified compounds at low concentrations were detected in the tested ethanol extract of *U. guianensis. In vitro* and *in vivo assays* have demonstrated that the pentacyclic oxindole alkaloids (POA) were the main source of the immunostimulant and cytotoxic activity of *U. tomentosa* (Heitzman et al., 2005; Winkler et al., 2004), being considered biochemical markers of this species and used to assure the quality of the plant material and commercial herbal medicines (Ganzera et al., 2001; Laus and Keplinger, 1994; Stuppner et al., 1992; Valente et al., 2006). However they are usually much more abundant in *U. tomentosa* than in *U. guianensis* (Pereira et al., 2008). Sandoval et al (2002) have demonstrated that the antioxidant and anti-inflammatory activities of the aqueous extract of the *U. guianensis* bark were independent of its alkaloid content. Another difference between these species was described by Valente et al., 2009. The TLC and HPLC-DAD-MS analysis of this ethanol extract from *U. guianensis*, but not from *U.*

tomentosa, revealed the presence of the flavonol kaempferol-3,7-O-(□)-dirhamnoside (kaempferitrin) as its major polyphenolic component.

Our results demonstrated that the ethanol extract from *U. guianensis* leaves has a low potential for systemic toxicity; similarly to its parental species *U. tomentosa* (Sheng et al., 2000). Physiological alterations regarding organ relative weights were observed. However, there were no signs of structural changes or of an enzymatic induction that suggested a toxic effect. Despite the similar water and food intake among the experimental groups, body weight gain was significantly reduced in animals treated with *U. guianensis*. This effect could be related to the flavonol kaempferitrin, already described in other plant species (Fang et al., 2005; Pinheiro et al.; 2006). A recent study demonstrated that kaempferitrin stimulates the secretion of adiponectin, an adipocyte-derived protein with a broad spectrum of biological activities, including oxidation of tissue fatty acid and reduction of serum lipid levels (Tzeng et al., 2009). These effects have been reported to promote weight loss without a significant reduction in food intake (Lang and Ratke, 2006; Tzeng et al., 2009). Thus, we hypothesized that the reduction in body weight determined by *U. guianensis* may be not associated with a systemic toxicity; but is rather a result from the activity of its major polyphenolic component, kaempferitrin, over the metabolic process. Furthermore, the dose-dependent monocytosis detected in blood samples obtained from animals treated with the *U. guianensis* could be also related to increased adiponectin levels triggered by kaempferitrin. Adiponectin was recently described as endowed with anti-atherogenic and anti-inflammatory properties, inhibiting the surface endothelial expression of VCAM-1, E-selectin and ICAM-1 induced by TNF-α (Ouchi et al., 2007). Inhibition of adhesion molecules expression at the endothelial cells could avoid transendothelial migration of monocytes to the tissues, resulting in the observed peripheral monocytosis.

Supporting this idea, a very recent report by Hwang et al. (2010) provided the evidence that an herbal extract from *Gardenia jasminoides* was able to inhibit TNF-α-induced vascular inflammation in endothelial cells.

Animals treated with *U. guianensis* also exhibited an enlargement of splenic marginal zone and more evident germinal centers, a region characterized by extensive proliferation of B cells which, when irrelevant or auto-reactive, undergo apoptosis (Allen et al., 2007). Since the number of B lymphocytes with the phenotype $CD45RA^+$ was at normal range, and the work was done with pathogen-free animals, this histological change could result from an enhanced viability of splenic B cells. This possibility is reinforced by both, the fact that IL-10 enhances the viability of purified splenic B cells (Levy and Brouet, 1994) and the ability of kaempferitrin to promote increased IL-10 production through the up-regulation of adiponectin synthesis (Pamuk et al., 2006). The phenotypic analysis of lymphocyte subsets also revealed that animals treated with the highest doses of the extract (500 and 1250 mg/kg) presented a slight increase in the number of $CD4^+$ T lymphocytes and a decreased number of immature lymphocytes with $CD4^+$ $CD8^+$ phenotype. It has been experimentally demonstrated that $CD4^+CD8^+$ lymphocytes are partially immunocompetent cells that undergo post thymic maturation at the periphery, becoming functionally mature $CD4^+$ T cells (Jimenez et al., 2002). Therefore, *U. guianensis* could stimulate the transition of $CD4^+CD8^+$ lymphocytes to its mature $CD4^+$ helper phenotype.

The data obtained from the *in vitro* protocol revealed distinct results that were dependent on *U. guianensis* concentration. Lower concentrations of the extract resulted in an increased viability of lymphocytes. On the other hand, higher concentrations of the extract determined a reduction in both, lymphocyte proliferation and cytokine production. Cytokines associated with Th1 (IL-2, IFN-γ and TNF-α) and with Th2 (IL-

5) profile were specially inhibited. These findings are coherent with the anti-inflammatory and anti-allergic properties previously described for this plant (Carvalho et al; 2006; Sandoval et al., 2002; Piscoya et al., 2001).

5. Conclusion

In summary, the ethanolic extract from *U. guianensis* leaves was not significantly toxic. Neverthless, some structural alterations in the spleen, the increased percentage of splenic $CD4^+$ T cells and peripheral monocytosis observed *in vivo* would require more detailed studies thereafter. The *in vitro* immunomodulatory effects, as seem by the inhibition of mitogen-induced production of Th1 cytokines and IL-5, reinforced the potential of the extract to treat inflammatory and allergic disorders.

Aknowledgements

This study has been supported by grants from FAPESP, FAPERJ, CAPES and CNPq. The authors would like to thank to Dr. João Lauro Viana de Camargo, supervisor of the laboratory Toxicam (Medical School, São Paulo State University), Dr. Peter May and N.G.O. Pro-Natura (Brazil) for the kind donation of the plant material, Dr. Hélida B. N. Borges from the Central Herbarium of the UFMT, Brazil and the technical support provided by Paulo C. Georgete and Paulo R. Cardoso during the experimental period.

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Prevention of experimental type 1 diabetes by *Uncaria tomentosa* extract: Th2 polarization, regulatory T cell preservation or both?

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Abstract

Type 1 diabetes mellitus is a Th1-like autoimmune disease with multifactorial etiology, characterized by an inflammatory mononuclear infiltrate in the islets and the progressive loss of insulin-secreting β-cells. Repeated injections of low doses of streptozotocin (MLDS) in susceptible strains of mice cause an autoimmune disease similar to human diabetes. In this study we investigated the immunomodulatory potential of *Uncaria tomentosa* (UT) aqueous-alcoholic on the progression of autoimmune diabetes induced by MLDS. **Methods:** C57BL/6 male mice were injected with five MLDS (40mg/kg) and orally treated with UT at 10-400 mg/kg during 21 days. Control groups received MLDS alone or the respective dilution vehicle. Clinical diabetes was defined as hyperglycemia higher than 200 mg/dL. Pancreatic mononuclear infiltrate and ß-cell insulin content was analyzed by HE and immunohistochemical staining, respectively, and measured by digital morphometry. Lymphocyte immunophenotyping and cytokine production were determined by flow cytometry analysis. **Results:** Treating the animals with 50-400 mg/kg of UT caused a significant reduction in the glycemic levels, as well as in the incidence of diabetes ($0.05<p<0.01$). The morphometric analysis of insulitis revealed a clear protective effect. Animals treated with UT at 400 mg/kg presented a higher number of intact islets and a significant inhibition of destructive insulitis ($p<0.05$). Furthermore, a significant protection against the loss of insulin-secreting presented β-cells was achieved, as observed by a careful immunohistochemical evaluation. The phenotypic analysis indicated that the groups treated with higher doses (100-400 mg/kg) presented $CD4^+$ and $CD8^+$ T-cell values similar to those observed in healthy animals. These same higher doses also increased the number of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cells ($p<0.05$). Moreover, the extract modulated the production of Th1 and Th2, with increased levels of IL-4 and IL-5 ($p<0.05$). **Conclusion:** The extract was effective to to prevent the progression of experimental autoimmune diabetes by different pathways.

Keywords: Uncaria tomentosa, type 1 diabetes, regulatory T cells, cytokine

1. Introduction

Type 1 diabetes mellitus (T1D) is a chronic autoimmune disease with multifactorial etiology and whose incidence is exponentially increasing in developed nations since the 1950s (Zipris, 2009). The early stage of the disease is characterized by an inflammatory mononuclear infiltration into pancreatic islets, with the progressive loss of insulin-secreting β-cells (Atkinson and Eisenbarth, 2001). Although both humoral and cellular mechanisms are activated during the progression of T1D, $CD4^+$ and $CD8^+$ T cells seem to be important final effectors of β-cell death (Willcox et al., 2009). It has been demonstrated that the severity of insulitis is closely related to an increased expression of pro-inflammatory cytokines such as IFN-γ, and TNF-α and a reduction in IL-4, IL-5 and IL-10 levels (Rabinovitch e Suarez-Pinzon, 2007). Furthermore, it is has been attributed an important the role for $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells on the regulation of immune tolerance and the self-reactive response during the development and progression of T1D in humans and rodents (Wing and Sakaguchi, 2010; van den Brandt et al., 2010).

In susceptible strains of mice, T1D with clinical and immunohistological features similar to those observed in humans can be induced by multiple low-doses of streptozotocin (MLDS) (Lin et al., 2010). One of the advantages of this model is the fact that hyperglycemia and insulitis are observed in a relatively short period of time (2-3 weeks after the last injection) in a high percentage of animals (Santos et al., 2009). Studies shown that diabetes development in this model is determined by the activity of self-reactive Th1 cells and macrophages and that IFN-γ is largely associated with disease progression (Cetkovic-Cvrlje e Uckun, 2005). Peripheral changes such as downregulation of Th2-derived cytokines and inhibition of Treg cells were also demonstrated in this disease model (Zdravkovic et al., 2009).

Ideal immune-based interventions for T1D should be initiated before the onset of the disease or, at least, during the period when a reasonable number of β-cells is still present in the pancreas. In this sense, herbal immunomodulators could provide an interesting tool to prevent or to reduce the destruction of islet cells through the dynamic modulation of immunological functions.

Uncaria tomentosa (Willd.) DC (*Rubiaceae*), also known as cat's claw, is a species native to the Amazon rainforest and surrounding tropical areas that is endowed with immunomodulatory properties. These properties include modulation of the cytokines TNF-α, IL-1β and IL-6 (Allen-Hall et al., 2010; Allen-Hall et al., 2007; Lemaire et al., 1999), enhancement of phagocytosis (Wagner et al., 1985), regulation of iNOS and NF-κB expression (Sandoval-Chacón et al., 2000) and the inhibition of lymphocyte proliferation (Winkler et al., 2004; Akesson et al., 2003). More recently, Reis et al. (2008) reported that the alkaloid fraction from *U. tomentosa* caused a strong inhibition of TNF-α and IFN-γ production by human monocytes that were infected with type-2 Dengue virus. So far, the chemical analysis of the species revealed the presence of indole and oxindole alkaloids in the whole plant. Previous studies have associated six pentacyclic oxindole alkaloids (POA) that are present in leaves, bark and roots to its immunomodulatory properties (Winkler et al., 2004; Wurm et al., 1998). Other components as proanthocyanidins, sterols, triterpenoids and carboxyl-alkyl esters were isolated from the bark (Akesson, et al., 2005; Gonçalves et al., 2005; Heitzman et al., 2005; Sandoval et al., 2000; Senatore et al., 1989). From these findings, some phytopharmaceuticals based on the bark ethanol extract and alkaloid-rich fraction from *U. tomentosa* have been standardized on the basis of their oxindole alkaloid content (Keplinger et al, 1989, 1994).

The present work was based on the hypothesis that the immunomodulatory properties of *U. tomentosa* could prevent β-cell mass loss and therefore retard T1D progression. For this purpose, we employed the classic MLDS diabetes model to test the effect of *U. tomentosa* extract on disease progression, cytokine production and number of peripheral $Foxp3^+$ Treg cells.

2. Material and Methods

2.1. Plant material and preparation of the extract

An aqueous-ethanol 1:1 extract that was previously obtained (Valente et al., 2006) from the stem bark of a wild specimen of *U. tomentosa*, collected in Cruzeiro do Sul, Acre, Brazil, and donated by Biosapiens Co., Brazil [voucher data in Miranda et al. (2003)], was used for the assays. The pentacyclic oxindole alkaloid (POA) profile and content of this extract was determined using high-performance liquid chromatography (HPLC) as previously described (Reis et al., 2008; Pereira et al., 2008). Briefly, the crude hydroalcoholic extract was treated with 0.1 N HCl and then partitioned with EtOAc. The resulting aqueous fraction was treated with NH_4OH to reach a pH of 9 to 10 and this fraction was then extracted with EtOAc. This alkaloid rich EtOAc fraction was dried and filtered, and the solvent evaporated under low pressure. A MeOH solution of this alkaloid fraction was injected into a reverse-phase HPLC system using a Lichrocart Lichrospher 5 µm, 125 x 4.6 mm i.d. column and a UV-detector at 245 nm under the same conditions previously described (Laus and Keplinger, 1994). Identification of the POA signals was done by comparing their relative retention times to those of pteropodine. The total alkaloid content was determined using HPLC-UV with the same chromatographic column and a mixture of 45% of MeCN and 55% of an aqueous 30 mM NH_4OAc solution as the mobile phase. The pH of the mobile phase was

adjusted between 6.8 and 7.0, and the analysis was run in isocratic mode with a flow rate of 1.0 mL/min at 60 ºC and detection at 245 nm. Five independent points for the external calibration curve were established using triplicate injections of an isopteropodine solution in MeOH. The curve presented linearity (R^2=0.9846) in the range of 4.75 to 76.0 μg/mL, with a standard deviation of 1.0 μg/mL. The minimum detectable concentration was estimated to be 1.0 μg/mL.

2.2. Animals

Six weeks old pathogen-free C57BL/6 male mice were purchased from the Multidisciplinary Center of Biological Investigations (CEMIB, Campinas, São Paulo, Brazil) and housed in controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 55 ± 20%, 12/12 h light-dark cycle and 4 daily exhaustion periods). All animals received Nuvilab CR-1 commercial food (Nuvital, Brazil) and water *ad libitum* and were submitted to a 2-week acclimation period before the experiment. Experimental protocols were approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, São Paulo, Brazil (protocol number 635).

2.3. Type 1 diabetes induction and experimental design

The animals were randomized in groups of eight, according to the protocol presented in Figure 1. T1D was induced by five intraperitoneal (i.p) injections of streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) at 40 mg/kg, during 5 consecutive days (Fallarino et al., 2010). *U. tomentosa* extract was given by gavage during 21 consecutive days, beginning at the first streptozotocin injection. A control group injected with the streptozotocin vehicle (citrate buffer, pH 4.5) was treated with the *U.*

tomentosa diluent (alcohol 10%). Blood glucose concentration was assessed on days 0, 12 and 21, using the Prestige LX Smart System Test-strips (Home Diagnostic Inc., Fort Lauderdale, USA). Glucose levels of 200 mg/dL (12 mmol/L) or above indicated diabetes development. After 21 days, the animals were euthanized and blood and tissue samples were collected.

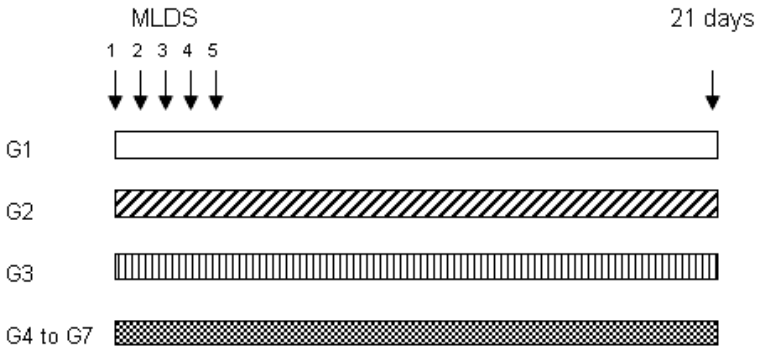

Figure 1 – Experimental protocol. T1D was induced by five sub-diabetogenic doses of streptozotocin or its dilution vehicle as follows. G1: alcohol 10% and citrate buffer; G2: U. tomentosa 400 mg/kg and citrate buffer; G3: MLDS 40 mg/kg and alcohol 10%; G4 to G7: MLDS 40 mg/kg and U. tomentosa at 10, 50, 100 or 400 mg/kg, respectively. n = 8 animals/group. U. tomentosa extract or its dilution vehicle alcohol 10% were adminestred by oral route. MLDS or its dilution vehicle citrate buffer were injected by intraperitoneal route.

2.4. Tissue processing and histological analysis

Pancreas, liver, kidneys, spleen, thymus, mesenteric lymph node and femur were removed and fixed in buffered formalin 10 % (phosphate buffer pH = 7.2) (Carlo Erba Reagents, Milano, Italy) for 24 hours. The spleen was divided into two portions: one for histology and the other for lymphocyte phenotypic analysis by flow cytometry. The paraffin blocks were cut into 5-µm-thick sections and stained with hematoxylin-eosin (HE) (Sigma-Aldrich, USA). The histophatological analysis was conducted following

previously published criteria by the Society of Toxicologic Pathology (Haley et al.,2005).

2.5. Morphometric analysis of pancreatic insulitis

A quantitative evaluation of islet mononuclear infiltration was conducted using a computer-assisted image system based on a Nikon Microphot-FXA optical microscope connected via a Sony Exwave HAD video camera to a computer. Total section area of each pancreas was measured to avoid any inter-animal variance. Further, islet and inflammation areas were measured individually, as described elsewhere (Kauri et al., 2007), using the KS300 software (Carl-Zeiss, AG, Germany). The result was expressed as the percentage of islet area showing mononuclear cell infiltration, according to the formula: mononuclear infiltrate area (μm^2) x 100 / islet area (μm^2). Finally, the results were categorized into the following score: 0 = intact islet; 1 = peri-insulitis ($\leq$ 10% of the islet infiltrated); 2 = moderate insulitis ($\leq$ 50% of the islet infiltrated); 3 = severe insulitis ($\geq$ 50% of the islet infiltrated); and 4 = destructive insulitis (Santos et al., 2009). At least 20 islets per pancreas were analyzed in non-consecutive sections.

To evaluate the protective effect of the extract against the loss of endocrine tissue, islets were manually traced and classified as small (300-2000 μm^2), medium (2000-10000 μm^2), large (10000-50000 μm^2) and very large (>50000 μm^2) (Kauri et al., 2007).

2.6. In-situ insulin detection

Islet insulin content was evaluated by the avidin-biotin peroxidase complex (ABC) method as previously described (Pinheiro et al., 2003). Briefly, deparaffinated 5-µm tissue sections on poly-L-lysine-coated slides were treated with 0.01 M citrate buffer

(pH 6.0) and heated twice, 5 min each time, in a microwave oven. To block nonspecific background staining, the sections were treated with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min and non-fat milk for 60 min. The slides were then incubated with polyclonal guinea pig anti-insulin antibody (dilution 1:500, Dako Glostrup, Denmark) overnight and then with rabbit anti–guinea pig IgG coupled with peroxidase (Dako, Glostrup, Denmark). Chromogen color development was accomplished with 3,3′-diaminobenzidine tetrahydrochroride (Sigma). The slides were counterstained with Harris's hematoxylin.

The morphometric analysis was performed using computer-assisted image system, as described at the item 2.5. Islet insulin content was established by calculating the area of immunopositive insulin relative to the islet's area, according to the formula: insulin positive area (μm^2) x 100/islet area (μm^2).

2.7. Splenic single cell suspensions

Spleens were pushed against a 50 µm sterile nylon mesh (BD, New Jersey, USA) in Petri dishes containing RPMI-1640 culture medium (Cultilab, Campinas, Brazil) and the erythrocytes were lysed with an isotonic solution of ammonium chloride, potassium bicarbonate and ethylenediamine tetraacetic acid (pH 7.2) (Sigma-Aldrich, USA). Cell suspensions were washed twice (200 x g / 10 min), and the pellet was resuspended in RPMI-1640 culture medium that was supplemented with 10% inactivated bovine fetal serum (Cultilab, Campinas, Brazil). Cellular viability was checked using trypan blue (Hyclone, Thermo Scientific, USA) and was considered adequate for values greater than 90 %. Cell suspensions were adjusted to 5 x 10^6 cells/mL for subsequent phenotypic analysis.

2.8. Lymphocyte immunophenotyping by flow cytometry

Splenocyte suspensions, obtained as described at the item 2.7, were adjusted to $1x10^6$ cells/mL and aliquots (100 µL) were transferred to 12x75 mm polypropylene round bottom test tube (BD Pharmingen, San Diego, CA, USA). The samples were incubated in the dark with FITC rat anti-mouse CD4 (L3T4-clone) and PE-Cy5 rat anti-mouse CD8a (Ly-2 clone) (BD Pharmingen, San Diego, CA, USA). After 30 min, the samples were washed twice (200 x g during 10 minutes) and resuspended in 400 µL of cold flow cytometry staining buffer (BD Pharmingen, San Diego, CA, USA). For the analysis of regulatory T cells, 100 µL of the splenocyte suspension ($1x10^6$ cells/mL) were incubate with FITC rat anti-mouse CD4 and PE-Cy7 rat anti-mouse CD25 following the same conditions described above. After the washing step, the pellet was submitted to a fixation/permeabilization solution (Ebioscience, San Diego, CA, USA) according to the manufacture's instruction and incubated in the dark for 30 min with PE labeled anti-mouse Foxp3 (Ebioscience, San Diego, CA, USA). The samples were washed twice (200 x g, 10 min) and the pellet was resuspended in 400 µL of cold flow cytometry staining buffer. Lymphocyte subsets were determined by FACS Calibur analysis (BD, New Jersey, USA), with gating on the total lymphocyte population. For consistency, a standard calibration bead (BD Pharmingen, New Jersey, USA) was used to set the forward scatter, side scatter and PMT voltage. For each experimental sample, a corresponding isotype control was used to set gates or positive/negative cell populations. The data were represented as the percentage of positive marked cells in their respective gates.

2.9. Th1 and Th2 cytokine production

Splenocytes obtained as described at the item 2.7 were dispensed in a 48-wells flat bottomed tissue culture plate (Corning, Costar, USA) at 5 x 10^6 cells/mL (450 µL). The samples were stimulated with Con A (10 µg/mL) and the plates were incubated for 48 h (37 °C, 5% CO_2). The supernantants were then collected and stored at -80° C. Th1 and Th2 cytokines were quantified using a murine Th1/Th2 cytometric bead array (CBA) kit according to the manufacturer's instructions (BD Pharmingen, USA). The FACSCalibur flow cytometer (BD Pharmingen, USA) was calibrated using setup beads, and a total of 3000 events were acquired for each sample. Individual cytokine concentrations (pg/mL) were evaluated by their fluorescence intensities and analyzed based on the standard reference curve of CELLQUEST and CBA software (BD Pharmingen, USA).

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Data were tested for normality (Kolmogorov-Smirnov) and homogeneity (Bartlett's) and, if necessary, they were submitted to appropriate transformations. Body weight, water and food intake, organ weight, hematology, immunophenotyping, cytotoxicity and cytokine concentration were compared by ANOVA. When ANOVA results were significant ($p < 0.05$), the comparison was followed by the Tukey test. For the non-parametric cases, the Kruskal-Wallis test was applied. Glycemic levels were analyzed by Friedman test.

3. Results

3.1. U. tomentosa reduced the incidence of diabetes

As expected, inoculation with MLDS was associated with a successful diabetes induction, characterized by high glucose concentrations. Mice injected with MLDS presented glycemic levels significantly higher at experimental days 12 and 21. Treatment with 80, 100 and 400 mg/kg of the *U. tomentosa* extract determined a significant reduction in the glycemic levels at the end of the experimental period (Figure 2a).

All tested doses (10 to 400 mg/kg) promoted a delay in diabetes incidence during the experimental period. Furthermore, the highest *U. tomentosa* dose (400 mg/kg) completely abrogated diabetes incidence at day 12 and also prevented raise of glycemic levels above 200 mg/dL in most of the animals at day 21 (Figure 2b).

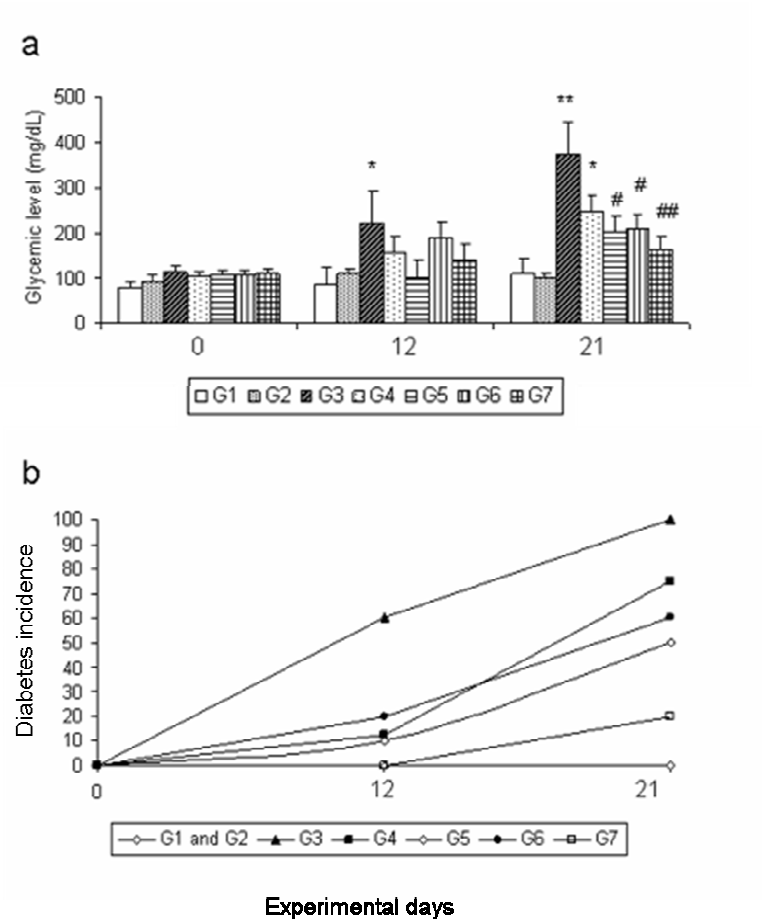

*Figure 2 – Effect of U. tomentosa on glycemic levels and diabetes incidence. a) U. tomentosa aqueous-ethanol extract determined a significant reduction of glycemic levels (Mean ± SD). * and **: Significantly higher than the observed in G1 and G2 groups; p<0,05 and p<0.001, respectively. # and ##: Significantly lower than the observed in G3 group; p<0.01 and p<0.001, respectively. b) U. tomentosa aqueous-ethanol extract delayed diabetes incidence. Values are expressed as percentage of diabetic animals. G1 and G2: negative control and U. tomentosa 400 mg/kg, respectively; G3: MLDS control; G4 to G7: MLDS + U. tomentosa at 10, 50, 100 and 400 mg/kg, respectively.*

3.3. Morphometric analysis of insulitis and insulin content

Animals injected with MLDS presented a clear pattern of insulitis, with the predominance of moderate (grade 2) and invasive (grade 3) insulitis. Treatment with the higher dose of the *U. tomentosa* extract (400 mg/kg) determined a significant protection against mononuclear infiltration, characterized by a higher percentage of intact islets

(grade 0) and a concomitantly reduction in the percentage of moderate, severe and destructive insulitis (Figure 3a).

As expected, insulin production by β-cells was clearly reduced in animals injected with MLDS (Figure 3b). However, *U. tomentosa* treatment, particularly at 400 mg/kg, promoted higher insulin content *in situ* (Figures 3c, 3d, 3e).

This higher dose also prevented reduction in islet size determined by MLDS, resulting in a predominance of medium and large islets (Table 1). Reduced frequency of apoptotic bodies, cytoplasmatic vacuolization and eosinophilia was also observed, reinforcing a protective effect against destructive insulitis (data not showed).

Table 1 – Islet size[1] in C57BL/6 mice injected with MLDS and orally treated with an aqueous-ethanol extract of *U. tomentosa* during 21 days

	Small (<2000μm²)	Medium (<10000μm²)	Large (<50000μm²)	Very Large (>50000μm²)
G1- Negative control	0	15	75	10
G2 – UT 400 mg/kg control	0	20	66	14
G3 – MLDS control	42	48	10	0
G4 – MLDS + UT 10 mg/kg	25	61	14	0
G5 – MLDS + UT 50 mg/kg	16	67	17	0
G6 – MLDS + UT 100 mg/kg*	0	40	48	12
G7 – MLDS + UT 400 mg/kg*	0	15	71	14

[1]*Islet size was measured using a computer-assisted image system and expressed as relative values (%) for each experimental group. * Groups G6 and G7 presented islet size significantly higher than the observed in G3 group (p<0.001 – chi-square test).*

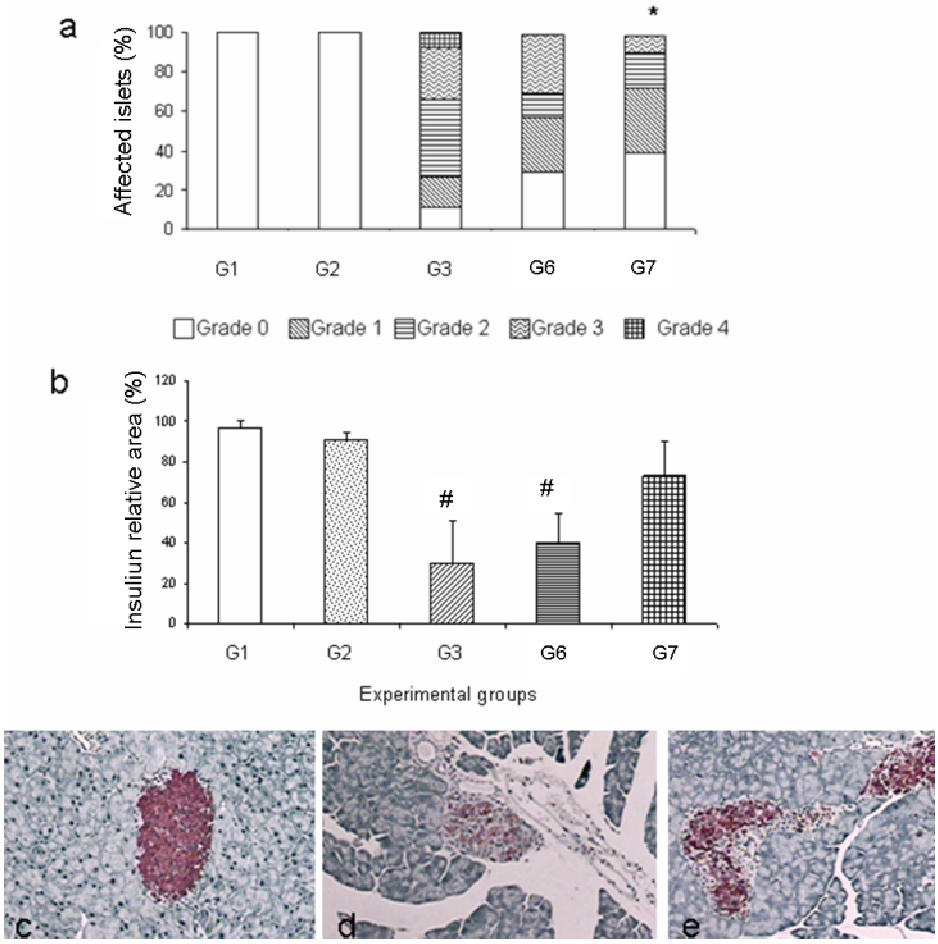

*Figure 3 – Protective effect of U. tomentosa aqueous-ethanol extract on the incidence of insulitis (a) and insulin production (b). * : Higher number of intact islets (Grade 0) when compared to G3 group ($p<0.05$, chi-square test). #: Significantly different from G1 and G2 control groups ($p<0.001$). Figures c, d and e indicated in situ insulin content. c: G1 control group, d: G3 MLDS group and e: G7 U. tomentosa 400 mg/kg group, respectively. G1 and G2: negative control and U. tomentosa 400 mg/kg, respectively; G3: MLDS control; G6 and G7: MLDS + U. tomentosa at 100 and 400 mg/kg, respectively.*

3.4. Phenotypic analysis of splenic lymphocyte subsets

Treatment with *U. tomentosa* at 100 and 400 mg/kg determined a significant increase in the number of $CD4^+$ T cells. The phenotypic analysis did not reveal significant difference regarding the percentage of $CD8^+$ T cells among the groups (Table 2).

Injection of MLDS also reduced the percentage of $CD4^+$ T lymphocytes and $CD4^+CD25^+Foxp3^+$ Treg cells. However, treatment with *U. tomentosa* at 100 and 400 mg/kg resulted in Treg cell values similar to those observed in the control groups (Table 1).

Table 1 - Phenotypic analysis[1] of splenic lymphocyte subsets in C57BL/6 mice injected with MLDS and orally treated with an aqueous-ethanol extract of *U. tomentosa* during 21 days

	$CD4^+$ cells	$CD8^+$ cells	$CD4^+CD25^+Foxp3^+$ Tregs
G1- Negative control	24.22 ± 1.1	15.2 ± 2.5	3.2 ± 0.3
G2 – UT 400 mg/kg control	23.0 ± 1.1	14.5 ± 0.9	3.0 ± 0.6
G3 – MLDS control	18.82 ± 0.6*	12.5 ± 0.4	2.3 ± 0.4*
G6 – MLDS + UT 100 mg/kg	24.26 ± 1.6	15.4 ± 3.5	2.7 ± 0.6
G7 – MLDS + UT 400 mg/kg	23.2 ± 1.5	14.9 ± 0.6	2.9 ± 0.5

[1] *Values are expressed as percentage of lymphocyte subsets (Mean ± SD), 10000 cells/animals were counted. * Significantly different from control, $p<0.05$.*

3.5. Effect of U. tomentosa aqueous-alcoholic extract on Th1/Th2 cytokine production

All animals injected with MLDS alone produced higher IFN-γ levels and a non-significant decrease of IL-4 and IL-5 levels, in comparison to the control groups. On the other hand, treatment with *U. tomentosa* determined a significant increase in IL-4 and IL-5 levels γ (Figure 4).

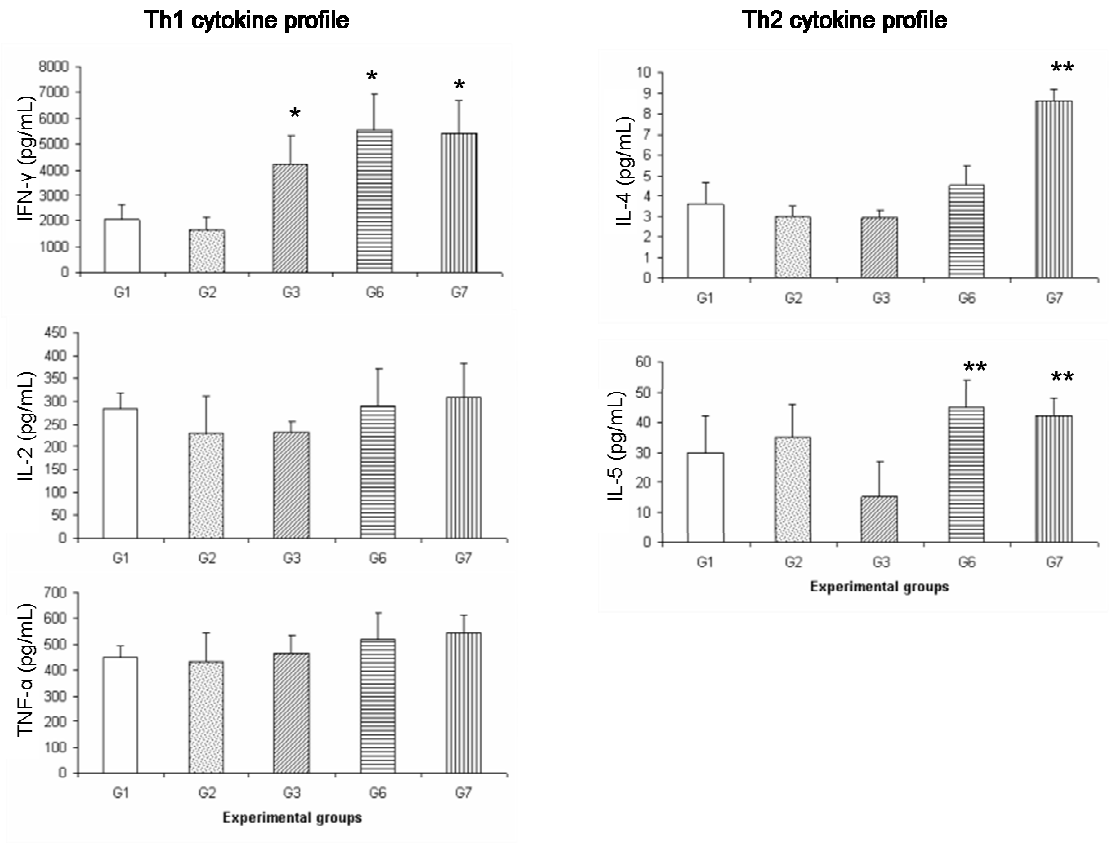

*Figure 4 – Effect of U. tomentosa aqueous-ethanol extract on the mitogen-induced Th1 and Th2 cytokine production. * Significantly different from G1 group, p<0.05; ** Significantly different from G3 group, p<0.05. G1 and G2: negative control and U. tomentosa 400 mg/kg, respectively; G3: MLDS control; G6 and G7: MLDS + U. tomentosa at 100 and 400 mg/kg, respectively.*

4. Discussion

Type 1 diabetes (T1D) is a chronic autoimmune disorder associated, in genetically susceptible individuals, with the generation and activation of autoreactive T cells. (Chentoufi et al., 2008). Experimental immunotherapies for T1D involve a broad spectrum of immunoregulatory strategies, including deletion of lymphocyte subsets or the use of substances that induce immune tolerance through T regulatory (Treg) cells activation (Goudy e Tisch., 2005). Some of these approaches even showed efficacy in

clinical trials, but risks such as abnormal cytokine release and/or reactivation of latent viruses render difficult its adoption (Luo et al., 2010). In this context, herbal immunomodulators could be considered potential adjuvant therapies for T1D. *Uncaria sp*, known as cat's claw, is being largely used as an antitumoral, antioxidant and immunomodulatory agent (Dreifuss et al., 2010; Allen-Hall et al., 2010). This study is the first demonstration that an aqueous-ethanolic *U. tomentosa* extract was able to prevent the full evolution of experimental diabetes. As a diabetes model we used C57BL/6 mice experimentally injected with multiple low streptozotocin doses (MLDS diabetes). This procedure is described as able to initiate a minor chemical destruction of β cells and shedding of islet's self-antigens. As a consequence, immune cells respond to ß-cell self-antigens and the progression of hyperglycaemia and insulitis evolves during a 2-week period (Cvjeticanin et al., 2010). This MLDS model has been largely employed to study T1D, including its immunopathogenesis and also therapeutic aspects (Karabatas et al., 2005; Kanitkar et al., 2008).

Many parameters sustained the protective effect of *U. tomentosa*. We initially observed that all tested doses were able to decrease glycemic levels. This effect was already observed at experimental day 12 but was more pronounced at day 21. Moreover, this activity was dose-dependent, being the highest concentration the most efficient one. Notably, the highest *U. tomentosa* dose (400 mg/kg) was able to reduce the diabetes incidence to 15%, whereas the STZ non-treated group displayed a 100% incidence. Similar hypoglycemic activity has been attributed to other plant-derived natural products, such as *Cassia auriculata* and *Bauhinia forficate* (Gupta et al., 2010; da Cunha et al., 2010). This protective potential has been associated with diverse mechanisms as restoration of pancreatic function, increase in insulin output and inhibition of glucose intestinal absorption. However, only a few of these evaluations

were done with experimental models of type 1 diabetes (Cvjetićanin et al., 2010; Beppu et al., 2006; Kobayashi et al., 2002).

The protective effect of *U. tomentosa* on type 1 diabetes was, at least partially, due to a diminished islet inflammatory process. Mice treated with the two highest *U. tomentosa* doses (100 and 400 mg/kg) were protected from developing the most severe inflammatory scores. Control mice injected with STZ presented a pattern of severe insulitis, characterized by predominance of moderate to invasive insulitis. Treatment with *U. tomentosa* changed this pattern by increasing the percentage of intact islets. A reduced frequency of parameters usually linked to islet disfunction, such as apoptotic bodies, vacuolization and eosinophilia, was also observed after *Uncaria* treatment. Interestingly, this extract also preserved much of the ability of the islet to produce insulin, as demonstrated by an *in situ* immunohistochemistry analysis. The tight association between pancreatic infiltration of the major orchestrators of β-cell damage, such as auto-reactive $CD4^+$ and $CD8^+$ lymphocytes, and destruction of insulin-secreting β-cell was recently reported (Wang et al., 2010).

Type 1 diabetes has been classically described as a predominant Th1 mediated disease, characterized by an elevated production of IFN-γ and IL-2 in both, human patients and experimental models (Csorba et al., 2010). In this context, it has been assumed that a naturally predominant Th2 profile or a procedure able to induce this profile, can avoid or ameliorate diabetes manifestations (Christen and von Herrath, 2004; Muller, 2002). In this investigation we also confirmed this Th1 polarization in STZ injected mice by evaluating cytokine production in spleen cells stimulated with ConA. Cell cultures from diabetic mice produced higher IFN-γ levels than normal animals. No differences were observed in IL-4 levels. Treatment with *U. tomentosa* did not modify the high IFN-γ levels but the highest extract dose significantly increased

both IL-4 and IL-5 levels. These results suggested, therefore, that the observed protective effect could be due, at least partially, to a Th2 polarization. In fact, previous studies have demonstrated the potential of herbal extracts to modulate the incidence and progression of type 1 diabetes through the dynamic modulation of cytokines toward a Th2 profile (Li et al., 2010; Sa et al., 2007).

As $CD4^+CD25^+$Foxp3 regulatory T cells (Treg cells) are essential to maintain self-tolerance (Wan, 2010) and have also been a promising target in autoimmunity therapy (Hsieh and Bautista, 2010), its possible contribution to the protection determined by *Uncaria* was evaluated. Our data showed that STZ-diabetic mice presented a significant reduction in the percentage of spleen $CD4^+$ T cells and that this finding was concomitantly associated with an also striking decrease in the percentage of $CD4^+CD25^+$Foxp3 Treg cells in this lymphoid organ. Treatment with *U. tomentosa* extract restored normal levels of these Treg cells in the spleen. Our interpretation of this data is based on evidences that Treg cells dislocate from secondary lymphoid organs to the target inflammed organ or tissue to control the inflammatory process (Kim, 2006). In this context, we could first hypothesize that natural Treg cells, usually present in the spleen, dislocated to the pancreas but were not able to control inflammation due to their small amount and/or activation status. In this same line of thought, *Uncaria tomentosa* could, by determining an increased percentage of Treg cells, partially control insulitis in MLDS diabetes and also restore the normal Treg level in the spleen. This possibility is, at some extension, supported by literature. Contribution of Treg cells has indeed been described as able to control insulitis in the MLDS model (Zdravkovic et al., 2009). In addition, it was demonstrated in the NOD mice model that peripheral Treg cells were able to prevent diabetes by initially homing to the pancreas (Lepault and Gagnerault, 2000).

It is important to highlight that this treatment, even at the highest extract doses, was not associated with any signs of systemic or organ-specific toxicity. These finding are sustained by a previous work that demonstrated a LD50 higher than 8 g/kg for the aqueous extract from *U. tomentosa* (Sheng et al, 2000).

Even thought this investigation had employed the whole *U. tomentosa* bark aqueous-ethanol extract, it is possible that most of the observed effects were mediated by its immunomodulatory alkaloid fraction (Reis et al., 2008; Winkler et al., 2004).

Taken together, our results indicate that *U. tomentosa* was able to significantly protect mice against type 1 diabetes development. The immunological assays indicated that this protective ability could be determined by a Th2 polarization, an expansion of Foxp3+ Treg cells or both. As this protection was devoid of any relevant associated deleterious effect, we believe that this extract or more purified derivatives are worthwhile to be considered in human diabetes treatment, and therefore, must be extensively studied.

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Immunotherapeutic effect of *Uncaria guianensis* ethanol extract on the development of experimental type 1 diabetes

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Abstract

Type 1 diabetes mellitus is a chronic autoimmune disease characterized by the infiltration of the pancreatic islets by inflammatory mononuclear cells and the progressive loss of insulin-secreting β-cells. Repeated injections of low doses of streptozotocin (MLDS) in susceptible strains of mice cause an autoimmune disease similar to human diabetes. In this study we investigated the immunomodulatory potential of *Uncaria guianensis* (UG) ethanol extract on the progression of MLDS diabetes. **Methods:** C57BL/6 male mice were injected with five MLDS (40mg/kg) and orally treated with UG at 100 and 400 mg/kg during 21 days. Control groups received MLDS alone or the respective dilution vehicle. Clinical diabetes was defined as hyperglycemia higher than 200 mg/dL. Pancreatic mononuclear infiltrate and insulin content was analyzed by HE and immunohistochemical staining, respectively, and measured by digital morphometry. Lymphocyte immunophenotyping and cytokine production were determined by flow cytometry analysis. **Results:** Treating the animals with 100 and 400 mg/kg of UG caused a significant reduction in the glycemic levels, as well as in the incidence of diabetes ($0.05<p<0.01$). The morphometric analysis of insulitis revealed that animals treated with UG at 400 mg/kg presented a higher number of intact islets and a significant inhibition of destructive insulitis ($p<0.05$). Furthermore, a significant protection against the loss of insulin-secreting presented β-cells was achieved, as observed by a careful immunohistochemical evaluation. The phenotypic analysis indicated that the groups treated with 100 and 400 mg/kg of UG presented $CD4^+$ and $CD8^+$ T-cell values similar to those observed in healthy animals. These same doses also increased the number of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cells ($p<0.05$). Finally, UG inhibited the production of the pro-inflammatory cytokine IFN-γ. **Conclusion:** Our results suggested that the therapeutic effect of UG could be related to its ability to reduce the levels of IFN-γ, followed by a potential to increase the number of regulatory T-cells.

Keywords: Uncaria guianensis, type 1 diabetes, regulatory T cells, cytokine

1. Introduction

Type 1 diabetes (T1D) is a chronic inflammatory disease characterized by the destruction of insulin-producing ß-cells through T-cell mediated mechanisms (Atkinson and Eisenbarth, 2001). In order to gain insight into T1D pathogenesis and to explore new therapeutic approaches, several animal models have been proposed (von Herrath and Nepom, 2009). In susceptible strains of mice, injection of multiple low doses of streptozotocin (MLDS) leads to the development of a condition with clinical and immunohistological features similar to those observed in the human disease. T cells and macrophages are thought to be involved in disease's progression through the synthesis of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1 and IL-17 (Hill and Rabinovitch and Suarez-Pinzon, 2007). These cytokines contribute to diabetes development by recruiting effector cells to the endocrine pancreas and by triggering the release of free-radicals. It is also demonstrated that the increased production of anti-inflammatory type 2 and 3 cytokines, such as IL-4, IL-10 and TGF-ß, correlates with protection from T1D (Rabinovitch and Suarez-Pinzon, 2007).

Ideal immune-based therapies for T1D must focus on preventing β-cell mass loss by controlling the autoimmune process. This intervention should be initiated before the onset of the disease or, at least, during the period when a reasonable number of β-cells is still present in the pancreas (Staeva-Vieira et al., 2007). In this sense, herbal immunomodulators could provide an interesting tool to prevent or to reduce the destruction of islet cells.

Uncaria guianensis (Aubl.) Gmel. (Rubiaceae), also known as cat's claw, is a large woody vine native to the Amazon rainforest and surrounding tropical areas. This species has been shown to demonstrated antioxidant and anti-inflammatory activities

(Valente et al., 2009), however few works have explored its pharmacological and toxicological properties. Sandoval et al. (2002) reported that an aqueous extract from the barks of *U. guianenis* displays the capacity to scavenge free-radicals, to quench lipid peroxidation and also to inhibit the mitogen-induced production of the pro-inflammatory cytokine TNF-α *in vitro.* Another study conduced by Carvalho et al. (2006) demonstrated that *U. guianensis* possesses effective anti-inflammatory and anti-allergic activities, both *in vivo and in vitro*. In this study, the ethanolic extract from leaves of *U. guianensis* reduced murine paw edema and pleural exudation, whereas inhibited LPS-induced nitric oxide and CXCL8 generation and OVA-induced IL-5 production by peritoneal macrophages and splenocytes. The anti-inflammatory property of the species is also demonstrated in clinical trials. Piscoya et al. (2001) described that an aqueous extract from the bark of *U. guianensis* was an effective therapy for patients with osteoarthritis of knee through the modulation of NF-κB pathway and TNF-α production. Moreover, a commercial extract containing an association of *U. guianensis* and *Lepidium meyenii* demonstrated potent chrondoprotective effect through the stimulation of insulin-like growth factor 1 (IGF-1) and by preventing the catabolic action of IL-1ß (Miller, 2006).

To date, phytochemical studies of *Uncaria* genus revealed indole and oxindole alkaloids (whole plant) (Laus e Keplinger, 2003), proanthocyanidins (bark) (Sandoval et al., 2002), flavonols (leaf and bark) (Alvarez et al., 1988) and triterpenoid glycosides (bark) (Alvarez et al., 1988; Yépez et al., 1991). More recently, Valente et al. (2009) demonstrated the presence of the flavonol kaempferol-3,7-O-(α)-dirhamnoside (kaempferitrin) in an ethanolic extract from leaves of *U. guianensis*, suggesting its potential as a chemical marker for this species.

The present work was conduced based on the hypothesis that the anti-inflammatory properties of *U. guianensis* could set it as a good candidate to prevent β-cell death and to retard T1D progression. Besides, hypoglycemic effect was already described for the flavonol kaempferitrin in other plant species (Jorge et al., 2004; de Sousa et al., 2004). In this sense, we employed the classic MLDS model of type 1 diabetes to evaluate the potential of the ethanolic extract from *U. guianensis* leaves to modulate Th1/Th2 derived cytokines and to maintain immune-tolerance through the preservation of peripheral regulatory T cells.

2. Materials and Methods

2.1. Preparation of the extract

The ethanol extract from *U. guianensis* leaves was prepared by exhaustive maceration at room temperature, as previously described (Carvalho et al., 2006). This extract (2 g) was partitioned between CH_2Cl_2/H_2O to yield an insoluble yellowish solid (27 mg) from the aqueous fraction, containing the glycosilated flavonol kaempferitrin (Figure 1), as previously described (Valente et al., 2009). It was also partitioned with n-hexane and MeOH/H_2O 9:1. The MeOH/H_2O fraction and the isolated kaempferitrin solution (1 mg/mL) were filtered through 0.45 µm nylon membranes and injected (20 µl) into the HPLC system equipped with a reverse-phase C18 column Lichrocart Lichrospher 5 µm, 250 x 4.6 mm i.d. (Merck). Elutions were performed in a gradient elution mode at a 0.8 mL/min flow: 10 min 10% solvent B (MeCN) in solvent A (H_2O with 0.1% HCOOH, pH 3), 10-23 min 10-40% solvent B in A and finally 12 min 40% solvent B in A. The detection was performed at 265 nm and the diode-array detector was set at an acquisition range of 200-600 nm. Its pentacyclic oxindole alkaloid (POA)

profile and content were achieved by HPLC-UV using an alkaloid enriched fraction obtained by acid-base partition of the ethanol extract. Thus, 100.4, 453.9 and 520.6 mg of the crude alcoholic extract were ultrasonically (15 min) solubilized in parallel with HCl 0.1N (0.15 mL/mg of the extract), and then partitioned with EtOAc (same volume of the HCl solution, three times). The resulting aqueous fractions were treated with NH_4OH until pH 9-10 and extracted with EtOAc (Valente et al., 2006). These alkaloid rich EtOAc fractions were then dried with anhydrous Na_2SO_4 and the solvent evaporated under low pressure at 37°C yielding 1.8, 17.9 and 12.2 mg (2.6 ± 1.1 %) of the dried alkaloid fractions, respectively. Solutions of 72.0, 89.5 and 61.0 µg/mL, respectively, were then made in MeOH and filtered through 0.45 µm nylon membranes and injected (10 µl) into the HPLC system. Elution was carried out with a mixture of 45% of MeCN/55% of aqueous 30 mM NH_4OAc solution, pH adjusted at 6.8-7.0, isocratic mode, flow rate of 1.0 mL/min, at 60ºC and detection at 245 nm. The alkaloids peaks were identified by retention time relative to isopteropodine (Pereira et al., 2008). POA samples were quantified by external standardization relative to mitraphylline.

2.2. Animals

Six week old, pathogen-free C57BL/6 male mice, were purchased from the Multidisciplinary Center of Biological Investigations (CEMIB, Campinas, São Paulo, Brazil) and housed under controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 55 ± 20%, a 12/12 h light-dark cycle and 4 daily exhaustion periods). All animals received Nuvilab CR-1 commercial food (Nuvital, Brazil) and filtered water *ad libitum* and were submitted to a 2-week acclimation period before the experiment. Experimental protocols were approved by the Committee for Ethics in Animal

Experimentation of the UNESP Medical School, São Paulo, Brazil (protocol number 635).

2.3. Type 1 diabetes induction and experimental design

The animals were randomized in groups of eight, according to the protocol presented in Figure 1. T1D was induced by five intraperitoneal (i.p) injections of streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) at 40 mg/kg, during 5 consecutive days (Fallarino etal., 2010). Control animals received the dilution vehicle (citrate buffer, pH 4.2). *U. guianensis* or its dilution vehicle (ethanol 10%) was given by gavage during 21 consecutive days, beginning at the first streptozotocin injection. Blood glucose concentration was assessed on experimental days 0, 12 and 21, using the Prestige LX Smart System Test-strips (Home Diagnostic Inc., Fort Lauderdale, USA). Glucose levels of 200 mg/dL (12 mmol/L) or above indicated diabetes development. At the end of 21 days, the animals were euthanized and blood and tissue samples were collected.

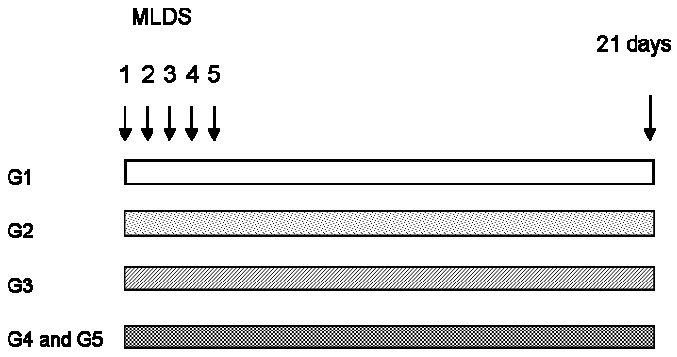

Figure 1 – Experimental protocol. T1D was induced by five sub-diabetogenic doses of streptozotocin or its dilution vehicle as follows: G1- ethanol 10% and citrate buffer; G2- U. guianensis 400 mg/kg and citrate buffer; G3 - MLDS 40 mg/kg and ethanol 10%; G4 and G5 – MLDS 40 mg/kg and U. guianensis at 100 or 400 mg/kg, respectively. n = 8 animals/group.

2.4. Tissue processing and histological analysis

Pancreas, liver, kidneys, spleen, thymus, mesenteric lymph node and femur were removed and fixed in buffered formalin 10 % (phosphate buffer pH = 7.2) (Carlo Erba Reagents, Milano, Italy) for 24 hours. The spleen was divided into two portions: one for histology and the other for lymphocyte phenotypic analysis by flow cytometry. The paraffin blocks were cut into 5-µm-thick sections and stained with hematoxylin-eosin (HE) (Sigma-Aldrich, USA).The histophatological analysis was conducted following previously published criteria by the Society of Toxicologic Pathology (Haley et al.,2005).

2.5. Morphometric analysis of pancreatic insulitis

A quantitative evaluation of islet mononuclear infiltration was conducted using a computer-assisted image system based on a Nikon Microphot-FXA optical microscope connected via a Sony Exwave HAD video camera to a computer. Total section area of each pancreas was measured to avoid any inter-animal variance. Further, islet and inflammation areas were measured individually, as described elsewhere (Kauri et al., 2007), using the KS300 software (Carl-Zeiss, AG, Germany). The result was expressed as the percentage of islet's area showing mononuclear cell infiltration, according to the formula: mononuclear infiltrate area (μm^2) x 100 / islet's area (μm^2). Finally, the results were categorized into the following score: 0 = intact islet; 1 = peri-insulitis ($\leq$ 10% of the islet infiltrated); 2 = moderate insulitis ($\leq$ 50% of the islet infiltrated); 3 = severe insulitis ($\geq$ 50% of the islet infiltrated); and 4 = destructive insulitis (Santos et al., 2009). At least 20 islets per pancreas were analyzed in non-consecutive sections.

To evaluate the protective effect of the extract against the loss of endocrine tissue, islets were manually traced and classified as small (300-2000 μm^2), medium (2000-

10000 μm^2), large (10000-50000 μm^2) and very large (>50000 μm^2) (Kauri et al., 2007).

2.6. In-situ insulin detection

Islet insulin content was evaluated by the avidin-biotin peroxidase complex (ABC) method as previously described (Pinheiro et al., 2003). Briefly, deparaffinated 5-µm tissue sections on poly-L-lysine-coated slides were treated with 0.01 M citrate buffer (pH 6.0) and heated twice, 5 min each time, in a microwave oven. To block unspecific background staining, the sections were treated with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min and non-fat milk for 60 min. The slides were then incubated with polyclonal guinea pig anti-insulin antibody (dilution 1:500, Dako Glostrup, Denmark) overnight and then with rabbit anti–guinea pig IgG coupled with peroxidase (Dako, Glostrup, Denmark). Chromogen color development was accomplished with 3,3′-diaminobenzidine tetrahydrochroride (Sigma). The slides were counterstained with Harris's hematoxylin.

2.7. Splenic single cell suspensions

Spleens were pushed against a 50 µm sterile nylon mesh (BD, New Jersey, USA) in Petri dishes containing RPMI-1640 culture medium (Cultilab, Campinas, Brazil) and the erythrocytes were lysed with an isotonic solution of ammonium chloride, potassium bicarbonate and ethylenediamine tetraacetic acid (pH 7.2) (Sigma-Aldrich, USA). Cell suspensions were washed twice (200 x g / 10 min), and the pellet was resuspended in RPMI-1640 culture medium that was supplemented with 10% inactivated bovine fetal serum (Cultilab, Campinas, Brazil). Cellular viability was checked using trypan blue (Hyclone, Thermo Scientific, USA) and was considered adequate for values greater than

90 %. Cell suspensions were adjusted to 5 x 10^6 cells/mL for subsequent phenotypic analysis.

2.8. Lymphocyte immunophenotyping by flow cytometry

Splenocyte suspensions, obtained as described at the item 2.7, were adjusted to 1x10^6 cells/mL and aliquots (100 µL) were transferred to 12x75 mm polypropylene round bottom test tube (BD Pharmingen, San Diego, CA, USA). The samples were incubated in the dark with FITC rat anti-mouse CD4 (L3T4-clone); PE-Cy5 rat anti-mouse CD8a (Ly-2 clone) (BD Pharmingen, San Diego, CA, USA). After 30 min, the samples were washed twice (200 x g during 10 minutes) and ressuspended in 400 µL of cold flow cytometry staining buffer (BD Pharmingen, San Diego, CA, USA). For the analysis of regulatory T cells, 100 µL of the splenocyte suspension (1x10^6 cells/mL) were incubate with FITC rat anti-mouse CD4 and PE-Cy7 rat anti-mouse CD25 following the same conditions described above. After the washing step, the pellet was submitted to a fixation/permeabilization solution (Ebioscience, San Diego, USA) according to the manufacture's instruction and incubated in the dark for 30 min with PE labeled anti-mouse Foxp3 (Ebioscience, San Diego, USA). The samples were washed twice (200 x g, 10 min) and the pellet was ressuspended in 400 µL of cold flow cytometry staining buffer. Lymphocyte subsets were determined by FACS Calibur analysis (BD, New Jersey, USA), with gating on the total lymphocyte population. For consistency, a standard calibration bead (BD Pharmingen, New Jersey, USA) was used to set the forward scatter, side scatter and PMT voltage. For the experimental samples, a corresponding isotype control was used to set gates or positive/negative cell populations. The data were represented as the percentage of positive marked cells in their respective gates.

2.9. Th1 and Th2 cytokine production

Splenocytes obtained as described at the item 2.7 were dispensed in a 48-wells flat bottomed tissue culture plate (Corning, USA) at 5 x 10^6 cells/mL. The samples were stimulated by mitogen Con A (10 µg/mL) and the plates were incubated for 48 h (37 °C, 5% CO_2). After that, the supernantants were collected and stored at -80° C. Th1 and Th2 cytokines were quantified using a murine Th1/Th2 cytometric bead array (CBA) kit according to the manufacturer's instructions (BD Pharmingen, USA). The FACSCalibur flow cytometer (BD Pharmingen, USA) was calibrated using setup beads, and a total of 3000 events were acquired for each sample. Individual cytokine concentrations (pg/mL) were evaluated by their fluorescence intensities and analyzed based on the standard reference curve of CELLQUEST and CBA software (BD Pharmingen, USA).

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Data were tested for normality (Kolmogorov-Smirnov) and homogeneity (Bartlett's) and, if necessary, they were submitted to appropriate transformations. Body weight, water and food intake, organ weight, hematology, immunophenotyping, cytotoxicity and cytokine concentration were compared by ANOVA. When the results of the ANOVA were significant ($p < 0.05$), the comparison was followed by the Tukey test. For the non-parametric cases, the Kruskal-Wallis test was applied. Glycemic levels were analyzed by Friedman´s test.

3. Results

3.1. U. guianensis reduced the incidence of diabetes

Animals injected with MLDS presented glycemic levels significantly higher than those observed in the control groups at experimental days 12 and 21. However, treatment with *U. guianensis* ethanolic extract at 100 and 400 mg/kg caused a significant reduction of mean glycemic levels and a delay in the incidence of hyperglycemia during the experimental period (Figures 2a and 2b).

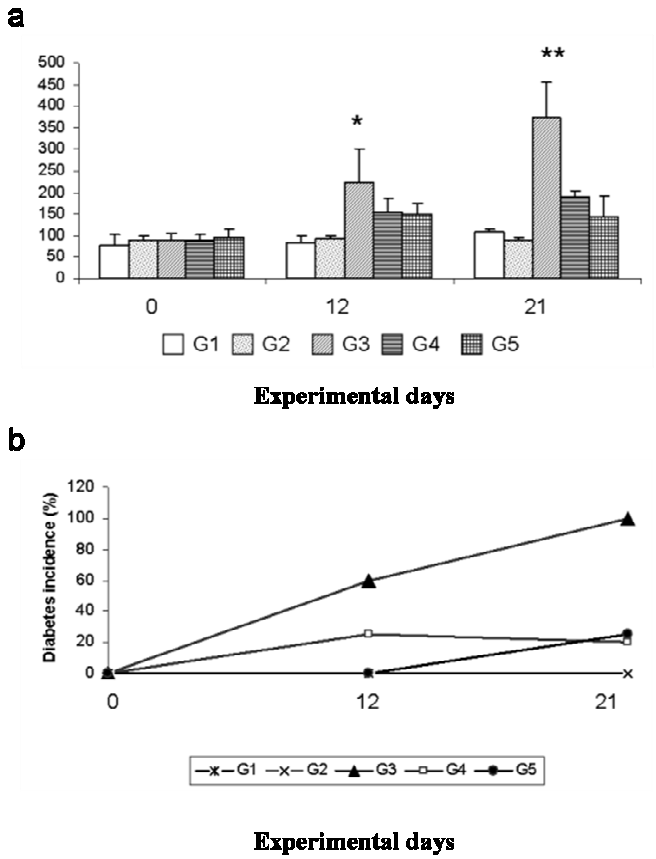

*Figure 2 – Effect of U. guianensis ethanol extract on the glycemic levels (a) and diabetes incidence (b) at experimental days 0, 12 and 21. G1 and G2: negative control and U. guianensis 400 mg/kg, respectively; G3: MLDS 40 mg/kg; G4 and G5: MLDS 40 mg/kg + U. guianensis at 100 and 400 mg/kg, respectively. The values are presented as Mean ± SD (a) or percentage (b). * and **: Glycemic levels significantly higher than the observed in G1 and G2 control groups; p<0,01 and p<0.001, respectively.*

3.3. Morphometric analysis of insulitis and insulin content

As expected, animals injected with MLDS presented a clear pattern of insulitis, with a predominance of moderate (grade 2) and invasive (grade 3) insulitis. Treatment with U. *guianensis* at 100 and 400 mg/kg resulted in a protective effect against the mononuclear infiltration, characterized by a significantly higher percentage of intact (grade 0) islets and a concomitantly reduction in the percentage of moderate, severe and destructive insulitis (Figure 3a).

The insulin production by pancreatic β-cells in the animals injected with MLDS was clearly reduced (Figure 3b). However, treatment with the highest tested dose of the extract (400 mg/kg) promoted higher insulin content *in situ* (Figure 3c, 3d e 3e).

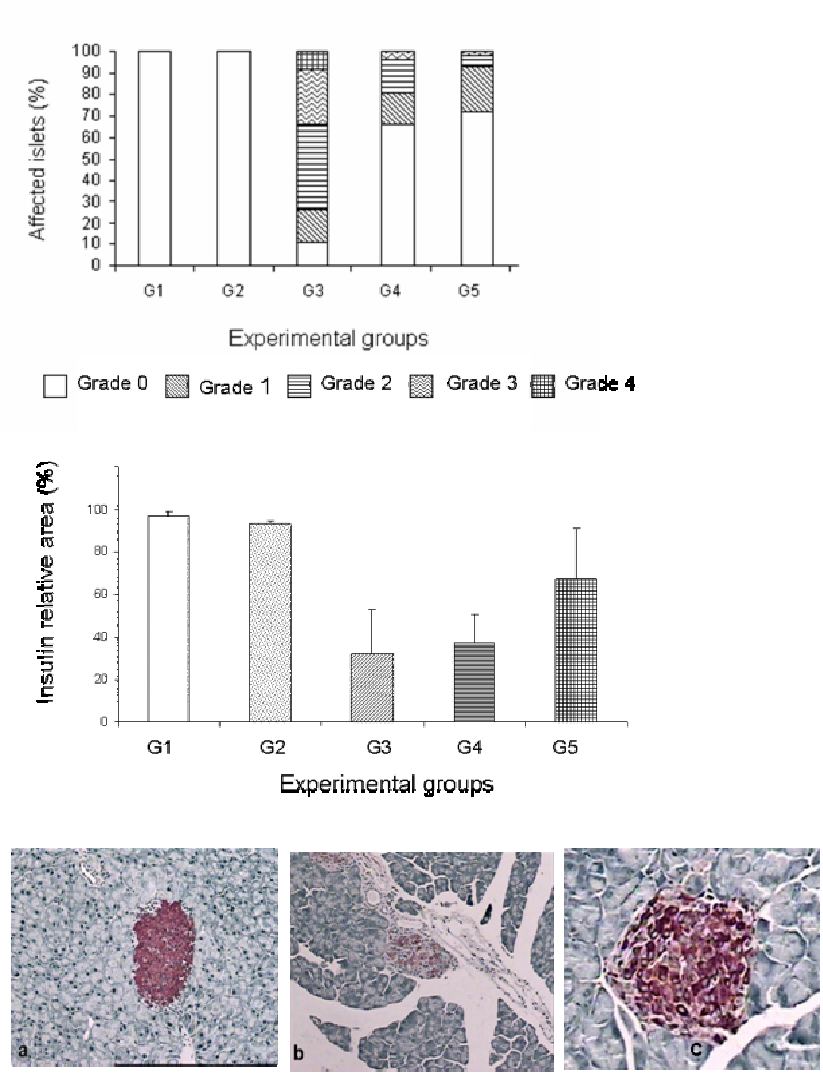

*Figure 3 – Protective effect of U. guianensis ethanol extract on the incidence of insulitis (a) and insulin production (b). * : Higher number of intact islets (Grade 0) when compared to G3 group (p<0.05, chi-square test). #: Significantly different from G1 and G2 control groups (p<0.001). Figures c, d and e indicated in situ insulin content. c: G1 control group, d: G3 MLDS group and e: G7 U. guianensis 400 mg/kg group, respectively. G1 and G2: negative control and U. guianensis 400 mg/kg, respectively; G3: MLDS control; G6 and G7: MLDS + U. guianensis at 100 and 400 mg/kg, respectively.*

3.4. Phenotipic analysis of splenic lymphocyte subsets

Animals injected with MLDS presented a decreased number of cells carrying the CD4 molecule. On the other hand, treatment with *U. guianensis* at 100 and 400 mg/kg resulted in $CD4^+$ T cell values similar to those observed in the control groups (Table 2).

The number of $CD4^+CD25^+Foxp3^+$ (Treg) lymphocytes in animals injected with

MLDS was significantly lower than the observed in the control groups. However, treatment with *U.guianensiss* at 100 and 400 mg/kg prevented the reduction of Treg cell observed during the progression of the disease (Table 2).

Table 2 – Phenotypic analysis[1] of splenic lymphocyte subsets in C57BL/6 mice injected with MLDS and orally treated with an ethanolic extract of *U. guianensis* during 21 days

	$CD4^+$ cells	$CD8^+$ cells	$CD4^+CD25^+Foxp3^+$ Tregs
G1- Negative control	21.72 ± 1.5	14.9 ± 2.1	3.3 ± 0.3
G2 – UG 400 mg/kg control	22.5 ± 0.6	14.2 ± 0.5	3.5 ± 0.6
G3 – MLDS control	18.82 ± 0.6*	12.5 ± 0.4	2.3 ± 0.4*
G6 – MLDS + UG 100 mg/kg	21.3 ± 1.8	13.9 ± 1.1	3.17 ± 0.3
G7 – MLDS + UG 400 mg/kg	21.6 ± 1.4	15.2 ± 1.7	4.0 ± 0.4

*1 Values are expressed as percentage of lymphocyte subsets (Mean ± SD), 10000 cells/animals were counted. * Significantly different from control, $p<0.05$.*

3.5. Effect of U. guianenis extract on Th1/Th2 cytokine production

Animals injected with MLDS presented a Th1 cytokine profile, characterized by significantly elevated IFN-γ and lower IL-4 and IL-5 levels. Treatment with the highest concentration of the *U. guianensis* extract prevented this Th1 polarization, determining a significant increase in IL-4 and IL-5 but not in IFN-γ levels (Figure 4).

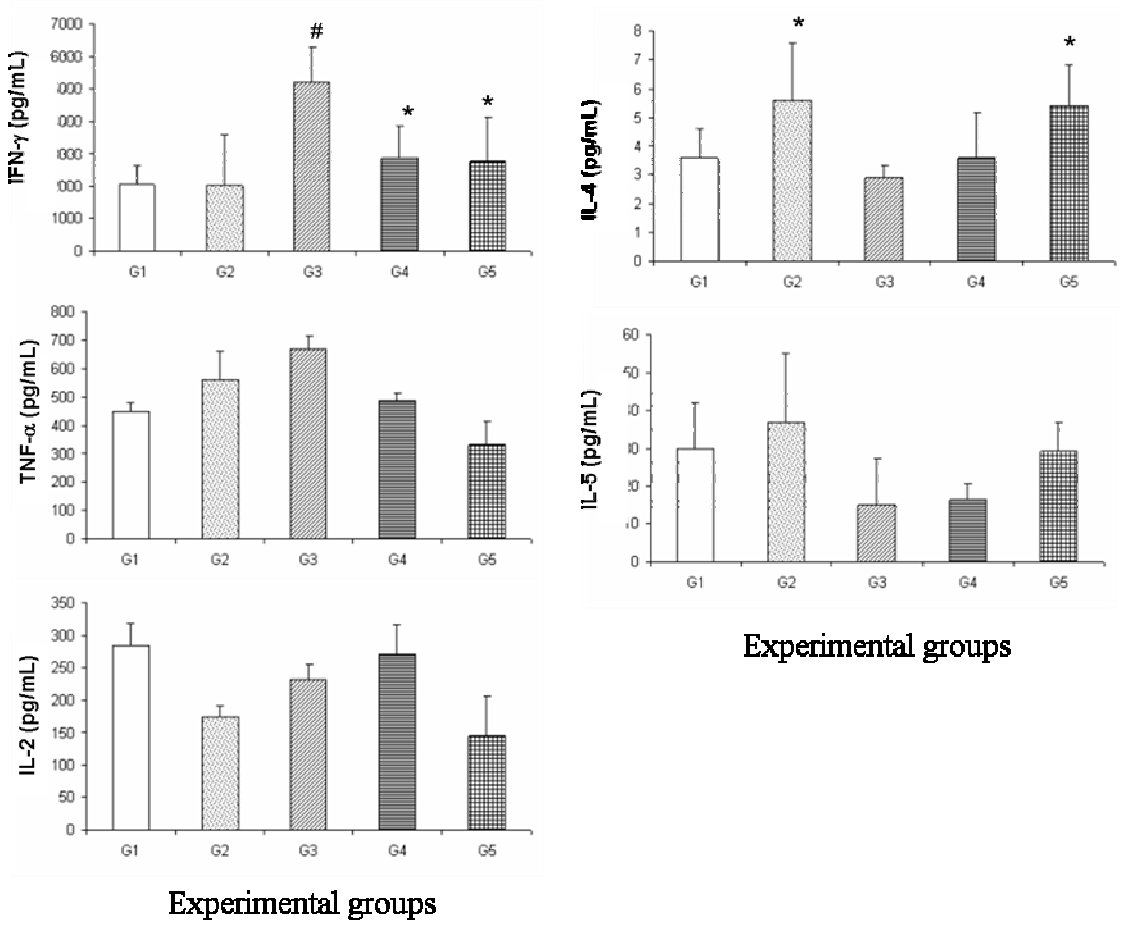

*Figure 4 – Effect of U. guianensis ethanol extract on the mitogen-induced Th1 (IFN-gamma, TNF-alpha and IL-2) and Th2 (IL-4 and IL-5) cytokine production. G1 and G2: negative control and U. guianensis at 400 mg/kg control groups, respectively; G3: MLDS diabetes control; G4 and G5: MLDS + U. guianensis at 100 or 400 mg/kg, respectively. * Significantly different from G3 group, p<0.05; # significantly different from G1 group, p<0.05.*

4. Discussion

In this study, we evaluated the potential of an authenticated ethanol extract from *U. guianensis* leaves to control the progression of T1D, a chronic autoimmune disease characterized by the mononuclear cell infiltration of pancreatic islets and the consequent loss of insulin-secreting ß-cells (Li et al., 2009). Aiming at this goal, we used the classic MLDS type 1 diabetes model, as the autoimmune process is initiated by minor chemical destruction of ß-cells and shedding of target autoantigens (Stosic-Grujicic et al., 2007).

The TLC and HPLC-DAD-MS analysis of this alcoholic extract indicated the presence of the flavonol kaempferol-3,7-O-(□)-dirhamnoside (kaempferitrin) as its major component. Five pentacyclic oxindole alkaloids (speciophylline with mitraphylline, pteropodine, isomitraphylline and isopteropodine) and other non-identified compounds at low concentrations were also detected. The presence of these pentacylic oxindole alkaloids was already described in *Uncaria tomentosa*, being considered a marker for this specie (Keplinger et al., 1999).

According to our initial hypothesis, the ethanol extract from leaves of *U. guianensis* could reduce the progression of T1D due to the presence of the flavonol kaempferitrin. Experimental studies have demonstrated that kaempferitrin has an insulinomimetic effect, as indicated by its ability to diminish blood glucose levels in the short-term (de Souza et al., 2004). This flavonol appears to possess pharmacological efficacy, since it induces a biological effect similar to that of insulin and is able to generate an insulin response at concentrations at the nanomolar range (Jorge et al., 2004). Interestingly, animals treated with our kaempferitrin-authenticated *U. guianensis* ethanol extract exhibited a clear protective effect against insulitis and also against the reduction of islet size. As consequence, higher insulin content *in situ* and lower glycemic levels were achieved.

To determine whether this protection could be related to the modulation of specific immunological pathways, we focused on the potential of *U. guianenis* to promote immunotolerance. Traditionally, Tregs lymphocytes are recognized as a mechanism by which the immune system maintains peripheral tolerance, since its depletion leads to the development of several auto-immune diseases (Tung et al., 2001). Previous studies have also correlated the reduced number of Tregs and diabetes development in the MLDS disease model (Bettini and Vignali, 2009; Zdravkovic et al.,

2009). Our results indicated that *U. guianensis* could reduce the incidence and progression of T1D not only by preservating the number of peripheral Tregs, but also by modulating Th1/Th2 cytokine imbalance. The higher number of $CD4^+$ T-cells associated with the inhibition of IFN-γ and the increase of IL-4 levels could suggest a cytokine polarization toward a protective Th2 profile. This finding is in agreement with the classical literature showing that IL-4 promotes the differentiation of Th2 cells *in vitro* through its activity on the transcription factor STAT6 and the up-regulation of GATA-binding protein (GATA3), the master regulator of Th2 differentiation (Cetkovic-Cvrlje e Uckun, 2005).

Based on the present results, it was demonstrated that *U. guianensis* ethanolic extract has the potential to reduce the severity of T1D by modulating the immune system toward a protective Th2 profile and also by a direct hypoglycemic effect caused by its major flavonol component kampferitrin.

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