Universidade Estadual Paulista

Instituto de Biociências - Campus de Botucatu

Programa de Pós Graduação em Ciências Biológicas
(Farmacologia)

Tese de Doutorado

Bidens pilosa L.: efeitos protetores na inflamação intestinal

Ana Elisa Valencise Quaglio

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Farmacologia) como parte das exigências para obtenção do título de Doutor

Orientador: Prof. Dr. Luiz Claudio Di Stasi

Botucatu

2015

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Quaglio, Ana Elisa Valencise.
Bidens pilosa L. : efeitos protetores na inflamação intestinal / Ana Elisa Valencise Quaglio. - Botucatu, 2015

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Luiz Claudio Di Stasi
Capes: 21001006

1. Intestinos - Doenças inflamatórias - Tratamento. 2. Bidens pilosa - Uso terapêutico. 3. Erva daninha. 4. Extratos vegetais.

Palavras-chave: Bidens pilosa; Inflamação; TNBS.

Auxílio Financeiro:

Conselho Nacional de Desenvolvimento Científico e Tecnológico

Fundação de Amparo à Pesquisa do Estado de São Paulo

Dedicatória

"... Muitas vezes basta ser: colo que acolhe, braço que envolve, palavra que conforta, silêncio que respeita, alegria que contagia, lágrima que corre, olhar que acaricia... amor que promove..."

(Cora Coralina)

Aos meus pais

A vocês que sempre me incentivaram a seguir meus sonhos nunca me deixando desistir... Vocês que sempre mostraram que a educação é o começo, meio e fim... Que abriram mão de muitas coisas pra propiciar a mim o melhor que eu poderia ter... Foram o colo que acolhe e o braço que envolve... Serão eternamente meu norte.

"Seus olhos meu clarão, me guiam dentro da escuridão... seus pés me abrem o caminho, eu sigo e nunca me sinto só... o meu melhor amigo é o meu amor..."

(Marisa Monte e Arnaldo Antunes)

Ao Eduardo,

A você que escolhi para caminhar comigo... Foram quatro anos de incertezas e dúvidas, mas com você ao meu lado tudo foi mais fácil, tudo foi mais tranquilo... Com você eu sigo e nunca me sentirei só...

A Deus, por me guiar, iluminar e me dar tranquilidade para seguir em frente com os meus objetivos e não desanimar com as dificuldades.

Aos meus irmãos Claudio e Juliano, cunhadas Juliana e Juliana e sobrinha Laura por sempre estarem do meu lado, me apoiando e me ajudando a nunca desistir dos meus sonhos.

A toda minha família, tios, primos e avó por sempre torcerem por mim e me apoiarem em todos meus momentos.

A minha nova família, Dona Odete, Eliane e Augusto pelo apoio e incentivo nesses quatro anos.

Aos meus amigos, de perto e de longe, pela paciência em alguns momentos de ausência e pelo incentivo constante.

Agradecimentos

A todos os amigos do laboratório – Alexandre (Kita), Alexandre (PV), Aline, Ellen, Fabiana, Lesvi e Tainan. Obrigada pelos ensinamentos, por ajudarem no meu crescimento, pela paciência e também pelos momentos de alegria durante os experimentos.

Obrigada Luiz por me ouvir e sempre me ajudar. Obrigada Celso pelos incontáveis e imensuráveis ensinamentos. Foram 4 anos de experimentos compartilhados, finais de semana trabalhando e muitos dados pra analisar mas tudo valeu a pena.

Aos amigos agregados do novo FitoFarmaTec obrigada pelo momentos de alegria tanto no laboratório quanto fora dele.

Ao Anthony, pela imensa paciência, pelos ensinamentos que nunca acabam e pela amizade.

Aos funcionários do departamento de Farmacologia – Cris, Paulão, Luiz e Janete por fazerem parte desta caminhada comigo.

A todos os funcionários da pós-graduação.

Aos professores da banca, pelo aceite e disponibilidade.

Ao CNPq pelo apoio financeiro.

A ChemyUnion pelo preparo e cessão do extrato de *Bidens pilosa* L.

"...ensinar não é transferir conhecimento, mas criar as possibilidades para sua própria produção ou a sua construção..." (Paulo Freire)

Ao prof. Dr. Luiz Claudio Di Stasi.

Já são 6 anos de confiança, ensinamentos constantes e muito aprendizado.

Obrigada por ser verdadeiramente um orientador para mim: aquele que orienta, direciona, guia.

Procuro semear otimismo e plantar sementes de paz e justiça. Digo o que penso, com esperança. Penso no que faço, com fé. Faço o que devo fazer, com amor. Eu me esforço para ser cada dia melhor, pois bondade também se aprende. Mesmo quando tudo parece desabar, cabe a mim decidir entre rir ou chorar, ir ou ficar, desistir ou lutar; porque descobri, no caminho incerto da vida, que o mais importante é o decidir.

Cora Coralina

Prólogo

Artigos Científicos

Aceitos

MARCELINO, M.Y.; FUOCO, N.L. ; QUAGLIO, A.E.V.; DE CAMARGO BITTENCOURT, R.A.; GARMS, B.C.; DA MOTTA CONCEIÇÃO, T.H. ; DI STASI, L.C.; RIBEIRO-PAES, J.T.. Cell therapy in experimental model of inflammatory bowel disease. Journal of coloproctology (Rio de Janeiro. Impresso), 2014.

Submetidos

QUAGLIO, A.E.V.; CASTILHO, A.C.S.; DI STASI, L.C.. Experimental evidence of MAP kinases gene expression on the response of intestinal anti-inflammatory drugs. **Life Sciences**

Revisão

Quaglio, A.E.V. and Di Stasi, L.C.D.. Complementary Medicine based in plant compounds for treatment and prevention of inflammatory bowel disease: a critical review. World Journal of Gastroenterology.

Prêmios e títulos

Certificado de Honra ao Mérito no XXIII Simpósio de Plantas Medicinais do Brasil (2014) com os trabalhos:

- Efeitos de *Bidens pilosa* L. sobre genes alvo na inflamação intestinal em ratos

- Avaliação da atividade curativa da *Pfaffia glomerata* na inflamação intestinal.

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Avaliadora de trabalhos no XXIII Congresso de Iniciação Científica da UNESP – Marília (2011)

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XXIII Simpósio de Plantas Medicinais do Brasil – Goiânia/GO (2014)

20 Congreso Latinoamericano de Farmacología y Terapéutica – La Havana/Cuba (2013)

8th Congresso of ECCO – European Crohn's and Colitis Organization – Viena/Áustria (2013)

XXII Simpósio de Plantas Medicinais do Brasil – Bento Gonçalves/RS (2012)

II Simpósio de Farmacologia da UNESP – Botucatu/SP (2012)

II Simpósio do Centro de Microscopia Eletrônica do IBB – Botucatu/SP (2011)

8th International Congresso f Pharmaceutical Sciences – Ribeirão Preto/SP (2011)

X Jornada Paulista de Plantas Medicinais e I Simpósio da Farmácia Verde – Assis/SP (2011)

I Simpósio de Farmacologia da UNESP – Botucatu/SP (2011)

Trabalhos apresentados

Apresentação oral

QUAGLIO, A.E.V.; COSTA, C.A.R.A.; ALMEIDA JR., L.D.; DI STASI, L.C.. Preventive and curative effects of Bidens pilosa L. in the TNBS model of intestinal inflammation. In: Latinfarma, 2013, Havana. Cuba

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Resumos publicados em revistas internacionais

QUAGLIO, A.E.V.; ALMEIDA JR., L. D.; COSTA, C.A.R.A.; DI STASI, L.C. . Effects of *Bidens pilosa* L. on the molecular targets in the TNBS-model of intestinal inflammation. In: 62 International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research GA2014, 2014, Guimarães. Portugal

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Eventos Internacionais

RIBEIRO, J.R.; MASAGO, F.; DEL BEN, A.; MACHADO, L.O.; QUAGLIO, A.E.V.; CHECON, J.; DIEMANT, G.; VELAZQUEZ, M.C.; DI STASI, L.C.. The role of *Bidens pilosa* L and *Physalis angulata* L in obese mice. In: Latinfarma, 2013, Havana. Cuba.

DEL BEN, A.; MACHADO, L.O.; RIBEIRO, J.R.; MASAGO, F.; QUAGLIO, A.E.V.; CHECON, J.; DIEMANT, G.; VELAZQUEZ, M. C.; DI STASI, L. C.. Preliminary evaluation of *Brassica campestris* and *Coffea arabica* effects in obesity. In: Latinfarma, 2013, Havana. Cuba.

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CURIMBABA, T.F.S.; CHAGAS, A.S.; ALMEIDA JR., L.D.; COSTA, C.A.R.A.; QUAGLIO, A.E.V.; HERCULANO, A.M.; LEAO, L.K.R.; BATISTA, E.J.O.; OLIVEIRA, K.R.M.; DI STASI, L.C.. Avaliação dos efeitos da dieta enriquecida com os frutos das palmeiras *Euterpe oleracea* Mart. e *Mauritia flexuisa* L.f na inflamação intestinal induzida por TNBS em ratos. In: XXIII Simpósio de Plantas de Medicinais do Brasil, 2014, Goiânia.

ALMEIDA JR., L.D.; CURIMBABA, T.F.S.; CHAGAS, A.S.; QUAGLIO, A.E.V.; COSTA, C.A.R.A.; DI STASI, L.C.. Avaliação dos efeitos preventivos da dieta enriquecida com farinha de banana verde (*Musa sp* AAA) no modelo experimental de doença inflamatória intestinal por TNBS. In: XXIII Simpósio de Plantas de Medicinais do Brasil, 2014, Goiânia.

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WITAICENIS, A.; CHAGAS, A.S.; ALMEIDA JR., L.D.; TANIMOTO, A.; QUAGLIO, A.E.V.; COSTA, C.A.R.A.; DI STASI, L.C.. A comparative study of the effects of esculetin, esculin and 4-methylesculetin in acute experimental model of inflammatory bowel disease. In: XXII Simpósio de Plantas medicinais do Brasil, 2012, Bento Gonçalves.

DEL BEN, A.; MACHADO, L.O.; RIBEIRO, J.R.; MASAGO, F.; QUAGLIO, A.E.V.; CHECON, J.; DIEMANT, G.. Análise dos efeitos de *Brassica compestris* e *Coffea arabica* sobre a obesidade induzida. In: XXII Simpósio de Plantas medicinais do Brasil, 2012, Bento Gonçalves.

RIBEIRO, J.R.; MASAGO, F.; DEL BEN, A.; MACHADO, L.O.; QUAGLIO, A.E.V.; CHECON, J.; DIEMANT, G.. Avaliação preliminar dos efeitos de *Bidens pilosa* L. e *Physalis angulata* L. sobre a obesidade induzida. In: XXII Simpósio de Plantas medicinais do Brasil, 2012, Bento Gonçalves.

MACHADO, L.O.; MASAGO, F.; DEL BEN, A.; RIBEIRO, J.R.; QUAGLIO, A.E.V.; CHECON, J.; DIEMANT, G.; VELAZQUEZ, M.C.; DI STASI, L.C.. Avaliação preliminar dos efeitos de *Coffea arabica* e *Physalis angulata* sobre a obesidade induzida por dieta hipercalórica. In: X Jornada Paulista de Plantas Medicinais, 2011, Assis.

DEL BEN, A.; RIBEIRO, J.R.; MACHADO, L.O.; MASAGO, F.; QUAGLIO, A.E.V.; CHECON, J.; DIEMANT, G.; VELAZQUEZ, M.C.; DI STASI, L.C.. Avaliação preliminar dos efeitos de *Brassica campestris* e *Bidens pilosa* L. sobre a obesidade induzida. In: X Jornada Paulista de Plantas Medicinais, 2011, Assis.

Formação complementar

Treinamento da PCR Quantitativa em tempo real (qPCR): uma abordagem teórico prática – São Paulo/SP (2013)

Mini-curso: “Métodos para avaliação farmacológica pré-clínica de plantas medicinais” – Bento Gonçalves/RS (2012)

Mini-curso: “Princípios em Microscopia Confocal” – Botucatu/SP (2011)

Seminário “Pipetas gilson: como aumentar a vida útil e obter melhores resultados” – Botucatu/SP (2011)

Mini-curso: “Controle de Qualidade de Matéria-prima vegetal para a indústria de cosméticos” – Assis/SP (2011)

Curso de férias “Avanços em Biologia Molecular” – Botucatu/SP (2011)

Disciplinas cursadas

Disciplinas	Créditos	Conceito
Estresse e Imunidade	4	A
Farmacologia Avançada	5	B
Tópicos Avançados em Imunologia e Imunopatologia	5	A
Classificação de Receptores Farmacológicos	3	A
Métodos de Purificação e Análise de Produtos Naturais	3	A
Abordagem experimental e computacional para investigar a estrutura e função de macromoléculas biológicas e suas interações	2	A
Tópicos de Atualização em Farmacologia: Mediadores da Resposta Inflamatória	12	A
Laboratório de Didática em Farmacologia	6	A
Total	**40**	

Organização de Eventos

- IV Simpósio de Farmacologia da UNESP (2014)

- I Curso de Inverno em Farmacologia e Biotecnologia – UNESP (2013)

- III Simpósio de Farmacologia da UNESP (2013)

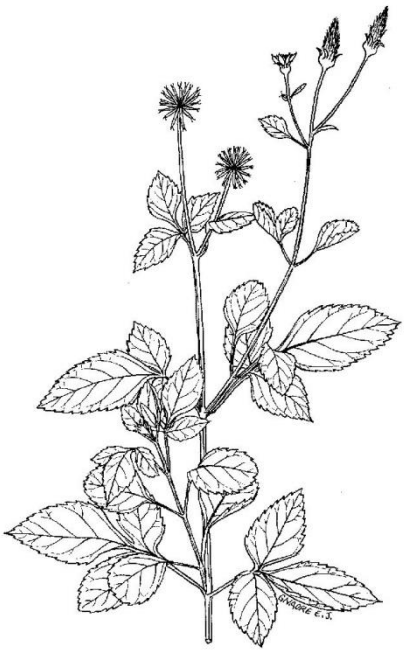
- II Simpósio de Farmacologia da UNESP (2012)

- I Simpósio de Farmacologia da UNESP (2011)

Resumo

A Doença Inflamatória Intestinal (DII) engloba, fundamentalmente, duas doenças distintas: a Doença de Crohn (DC) e a Colite Ulcerativa (CU), ambas caracterizadas por uma inflamação crônica do intestino, com períodos de exacerbação seguidos de intervalos prolongados de remissão dos sintomas. Apesar da DII ser objeto de pesquisa há várias décadas, a sua etiologia ainda é desconhecida e um único agente ou mecanismo isolado não parecem ser suficientes para produzir ou desencadear a doença. A dificuldade em se tratar de forma efetiva todos os pacientes com DII em função da baixa resposta aos fármacos associada a diversos efeitos colaterais corrobora a busca de novas fontes de compostos úteis no tratamento e prevenção da DII. Entre as várias estratégias disponíveis, a pesquisa de plantas medicinais se mostra promissora. Bidens pilosa L., família Asteraceae, é uma erva daninha com ampla distribuição e ocorrência em diversos países, sendo conhecida no Brasil como picão-preto. Apesar de ser considerada uma erva daninha sem importância econômica tem sido utilizada em várias populações com finalidade medicinal e sua aplicação em diversas enfermidades pode ser devido a sua composição química rica em phytol e ácido graxos poli-insaturados (ácido palmítico, ácido oleico ácido linoleico), compostos com efeitos anti-inflamatórios e/ou antioxidantes. No presente trabalho o extrato supercrítico de *Bidens pilosa* L. foi capaz de reduzir os níveis de mediadores pró-inflamatórios, como MPO, IL-1β e TNF-α além de modular fatores anti-inflamatórios como IL-10 tanto no protocolo preventivo quanto no curativo. Modulou também a expressão gênica de genes relacionados à inflamação e proteção da mucosa, como HSP70 e MUC. Além das alterações bioquímicas pôde-se notar uma melhora histológica em amostras de cólon analisadas por microscopia eletrônica. Associada a alterações estruturais ocorreu um aumento na produção e secreção de mucinas, agentes protetores da mucosa colônica. Dessa forma, o extrato de B.pilosa representa uma fonte importante de compostos que poderão ser utilizados no tratamento da DII em humanos.

Inflammatory Bowel Disease (IBD) comprises basically two distinct diseases: Crohn's disease (CD) and Ulcerative Colitis (UC), both characterized by chronic inflammation of the intestine, with exacerbation periods followed by long intervals of remission of symptoms. Despite being subject of research for decades, its etiology is still unknown and a single agent or isolated mechanism is not enough to produce or trigger the disease. The difficulty in effectively treat all patients based on the poor response to drug and in the several side effects that the treatments presents, supports the search for new sources of compounds useful in the treatment and prevention of IBD. Among the various strategies available, the research of medicinal plants shows promise. *Bidens pilosa* L., Asteraceae, is a weed with wide distribution and occurrence in some countries and is known in Brazil as picão-preto. Although it is considered a weed without economic importance, has been used in various populations with medical purpose and its application in various diseases may be due to its chemical composition rich in phytol and polyunsaturated fatty acid (palmitic acid, oleic acid and linoleic acid) compounds known to have anti-inflammatory and/or antioxidants effects. In the present study the supercritical extract of *Bidens pilosa* L. was able to reduce the levels of pro-inflammatory mediators, such as MPO, IL-1β and TNF-α associated with modulation of anti-inflammatory factors such as IL-10, both in the preventive and curative protocols. Also modulates gene expression of genes related to inflammation and mucosal protection, such as HSP70 and MUC. In addition to the biochemical changes might notice a histological improvement in colonic samples analyzed by electron microscopy. Together with structural changes was an increase in the production and secretion of mucin, mucosal protective agents. Thereby, *B.pilosa* extract is an important source of compounds which may be used in the treatment of IBD in humans.



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Introdução

A Doença Inflamatória Intestinal (DII) engloba, fundamentalmente, duas doenças distintas: a Doença de Crohn (DC) e a Retocolite Ulcerativa (RCU). Ambas caracterizam-se por uma inflamação crônica do intestino, com períodos de exacerbação seguidos de intervalos prolongados de remissão dos sintomas (ARSENEAU et al., 2007; ROSENSTIEL et al., 2009; STROBER; FUSS; MANNON, 2007), sendo marcada pela ulceração e infiltração de neutrófilos na mucosa, desconforto ou dor abdominal com hábitos intestinais alterados tais como diarreia e constipação (SINGH et al., 2003), mas com algumas diferenças quanto às características do processo inflamatório.

A Retocolite Ulcerativa é uma doença inflamatória intestinal restrita ao cólon e que dependendo da extensão e localização da inflamação pode ser classificada em proctite (parte inferior do cólon e reto), colite distal (cólon sigmóide com ou sem envolvimento do cólon descendente) ou pancolite (envolvimento de todo o cólon) (BAUMGART; SANDBORN, 2007). Histologicamente, a inflamação na retocolite ulcerativa se restringe a camada mucosa colônica, sendo caracterizada pela infiltração de linfócitos e granulócitos e presença de abscessos das criptas com infiltração de neutrófilos (COLLINS; CROITORU, 1994).

Diferente da retocolite ulcerativa, a Doença de Crohn é uma inflamação transmural do trato gastrointestinal (TGI) que pode afetá-lo por completo, desde a boca até o ânus. Normalmente se apresenta de forma descontínua em várias porções do TGI e pode promover complicações como estenoses, abscessos ou fístulas. Esta doença caracteriza-se ainda pela infiltração de linfócitos, formação de granuloma e fibrose (BAUMGART; SANDBORN, 2007; COLLINS; CROITORU, 1994).

Apesar da DII ser objeto de pesquisa há várias décadas, a sua etiologia ainda é desconhecida e um único agente ou mecanismo isolado não parecem ser suficientes para produzir ou desencadear a doença. A interação de fatores genéticos e ambientais (estresse, fatores dietários, uso de fármacos anticoncepcionais e anti-inflamatórios não esteroidais entre outros), em combinação com a microbiota intestinal, dispara um mecanismo que ativa células de origem imune e não-imune que compõem o sistema de defesa da mucosa intestinal, de modo que sua etiologia é considerada multifatorial (FIOCCHI, 1998). Por meio de uma resposta imune exagerada e inapropriada da mucosa à flora intestinal normal, a qual é facilitada por alterações na barreira epitelial intestinal e mediada principalmente por células T da mucosa, se desencadeia uma intensa síntese e liberação de diferentes mediadores pró-inflamatórios, incluindo espécies reativas de oxigênio e nitrogênio, eicosanóides, fator de agregação plaquetária e inúmeras citocinas pró-inflamatórias como TNF-α, IL-1β e IL-12.

Dados epidemiológicos demonstram que a retocolite ulcerativa é mais comum que a Doença de Crohn, sendo que a incidência e prevalência da DII variam amplamente devido às diferentes metodologias de estudo utilizadas, mas a maioria destes estudos mostra que a incidência e prevalência da RCU são duas vezes maiores que da DC (MARSHALL, 2008). Estudos retrospectivos realizados no início desta década mostram que a incidência e prevalência de DC foram estimadas em 6.9 e 133 por 100.000 habitantes, enquanto que para a RCU estas estimativas são de 8.3 e 299 por 100.000 habitantes (LOFTUS, 2004). No Brasil, um estudo realizado no Hospital das Clínicas de Botucatu mostrou que no período de 2001 a 2005 a incidência e a prevalência de RCU foram de 4.48 e 14.81 por 100.000 habitantes e de DC foi de 3.5 e

5.65 por 100.000 habitantes, sendo os dados de incidência semelhantes aos encontrados em estudos em países desenvolvidos (VICTORIA; SASSAKI; NUNES, 2009).

Diversos outros fatores estão associados a estas doenças, como o alto custo envolvido no tratamento. Dados recentemente publicados mostram que o custo médio anual/paciente para o tratamento da Doença de Crohn e Retocolite Ulcerativa nos Estados Unidos é respectivamente de US$ 8.265 e US$ 5.066 perfazendo um total aproximado de US$ 137 milhões/ano (KAPPELMAN et al., 2008). Outro fato é a característica incapacitante da DII restringindo o desenvolvimento normal das atividades diárias dos pacientes, gerando um alto impacto sobre sua qualidade de vida e nas atividades econômicas relacionadas (PEYRIN-BIROULET et al., 2010). Por último, mas não menos importante a Retocolite Ulcerativa e a Doença de Crohn estão associadas a um aumento na incidência de câncer colo-retal, sendo que, em um período de 30 anos, a chance de um paciente desenvolver esse tipo de tumor chega a 18% (JONES; SCOBEY; CHENG, 2014; TRIANTAFILLIDIS; NASIOULAS; KOSMIDIS, 2009).

O tratamento farmacológico dos pacientes com DII depende de diversos fatores, incluindo a localização e extensão da lesão, atividade da doença e o potencial envolvimento de outros órgãos. O correto diagnóstico dos diferentes tipos de DII também é um fator determinante para a escolha da medicação mais apropriada para cada um dos pacientes. Em adição, protocolos internacionais de tratamento farmacológico têm sido sugeridos considerando-se o tipo de DII que acomete o paciente (retocolite ulcerativa, Doença de Crohn e retocolite inespecífica), assim como a atividade da doença e sua intensidade (leve, moderada e grave).

Com base nestas características é possível a escolha do tratamento farmacológico mais apropriado, o qual pode incluir tanto o uso de drogas isoladas

como a combinação de diferentes fármacos. O tratamento padrão para DII inclui 5-aminosalicilato, glicocorticóides, imunosupressores e terapia biológica (GIRARDIN et al., 2012; NUNES et al., 2011), os quais normalmente têm como alvo eventos da cascata inflamatória (GIRARDIN et al., 2012; PITHADIA; JAIN, 2011). Porém, nenhum deles é específico para a doença e não existe, até o momento, cura para DII (BREITRUCK et al., 2013; FIOCCHI, 2012).

Os aminossalicilatos (5-ASA), sulfassalazina e mesalazina representam a primeira terapia de escolha no tratamento da doença inflamatória intestinal leve a moderada (DURICOVA et al., 2010). Apesar desta classe de medicamentos ser considerada o tratamento de primeira escolha, estas preparações têm o potencial de causar efeitos adversos em todo o organismo. Os efeitos mais comuns, que chegam a levar a intolerância, incluem dores de cabeça, dispepsia, náuseas, vômitos, anorexia e fadiga em até 45% dos pacientes (NAVARRO; HANAUER, 2003).

Os glicocorticoides tais como prednisolona são utilizados na terapia primária para DII moderada a severa, no entanto a dependência e os efeitos colaterais causados com o uso destes compostos influenciam diretamente a segurança e a tolerabilidade desses agentes (NAVARRO; HANAUER, 2003). JANI; REGUEIRO (2002) mostraram que a taxa de remissão em pacientes com RCU chegou a 75% para casos mais moderados e em casos mais severos a apenas 31% de remissão, porém a manutenção da remissão não é efetiva. Diversos estudos mostram que após um ano de tratamento, de 50% a 60% dos pacientes mantidos com glicocorticóides voltaram a apresentar a doença.

A eficácia dos análogos de purina Azatioprina e 6-mercaptopurina já está bem estabelecida na DII. A azatioprina é uma pró-droga rapidamente clivada a 6-mercaptopurina. Como produto resultante, a 6-mercaptopurina age como um

antagonista de purinas, inibindo a síntese de proteínas, RNA e DNA, dessa forma inibindo o crescimento celular (ARDIZZONE; PORRO, 2002). CUFFARI (2001) demonstrou que o tratamento com Azatioprina ou 6-mercaptopurina é eficaz em manter a remissão em 70% dos pacientes de DC dependentes de corticóides e é capaz de eliminar a necessidade de glicorticóides em aproximadamente 75% dos pacientes.

A terapêutica mais recente diz respeito ao uso da terapia biológica. Como TNF-α possui um papel importante no processo inflamatório de pacientes com DII, acredita-se que a inibição desta citocina possa ser um estratégia de tratamento poderosa em pacientes tanto com DC quanto RCU. Atualmente, a terapia anti-TNF inclui anticorpos monoclonais como infliximab, adalimumab e certolizumab (TRIANTAFILLIDIS; MERIKAS; GEORGOPOULOS, 2011). Diversos estudos analisam a taxa de sucesso da terapia biológica. Atualmente sabe-se que em torno de 60-65% dos pacientes respondem ao tratamento inicial com Infliximab e aproximadamente 33% conseguem entrar em remissão (LJUNG, 2004; PRESENT et al., 1999; TARGAN et al., 1997). Apesar da mediana taxa de sucesso do uso da terapia biológica, diversos estudos demonstram diferentes efeitos colaterais associados a este tipo de medicação, como por exemplo, tuberculose, linfomas e morte (GIES et al., 2010; LJUNG, 2004; POGGIOLI et al., 2007). Infelizmente, apesar de toda eficácia dos agentes biológicos no tratamento da DII, uma proporção significativa de pacientes não responde ou deixa de responder ao tratamento ao longo do tempo (YANAI; HANAUER, 2011).

A dificuldade em se tratar de forma efetiva todos os pacientes com DII em função da baixa resposta associada a diversos efeitos colaterais, fez com que surgisse a necessidade de se buscar por novas fontes de compostos úteis no tratamento da DII. Considerando-se os aspectos relacionados à sua etiologia, novas estratégias de

tratamento e prevenção da DII incluem moduladores da microbiota intestinal, agentes antioxidantes, e produtos naturais com diferentes mecanismos de ação.

Segundo o Ministério da Saúde (2006), a Organização Mundial de Saúde tem expressado a sua posição a respeito da necessidade de valorizar a utilização de plantas medicinais no âmbito sanitário, tendo em conta que 80% da população mundial utiliza estas plantas ou preparações destas no que se refere à atenção primária de saúde. Ao lado disso, destaca-se a participação dos países em desenvolvimento nesse processo, já que possuem 67% das espécies vegetais do mundo. O Brasil possui grande potencial para o desenvolvimento dessa terapêutica, como detentor da maior diversidade vegetal do mundo, ampla sociodiversidade, uso de plantas medicinais vinculado ao conhecimento tradicional e tecnologia para validar cientificamente este conhecimento (SAÚDE. et al., 2006). Além disso, o mercado mundial de fitoterápicos movimenta cerca de US$ 22 bilhões por ano. No Brasil, por exemplo, estima-se que o comércio de fitoterápicos seja da ordem de 5% do mercado total de medicamentos, avaliados em mais de US$ 400 milhões (PINTO et al., 2002).

Desta forma, a pesquisa de novos produtos naturais de origem vegetal representa um campo promissor na busca de substâncias ativas, sendo *Bidens pilosa* L., amplamente utilizada pela população para tratamento de inflamações no trato gastrointestinal, a espécie selecionada para o presente estudo. *Bidens pilosa* L., família Asteraceae, é uma erva daninha com ampla distribuição e ocorrência em países da África, América do Norte, Central e do Sul e Ásia (ABAJO et al., 2004; CHANG, C. L. et al., 2005; HORIUCHI; WACHI; SEYAMA, 2010; KVIECINSKI et al., 2008). No Brasil é conhecida como picão-preto. Apesar de ser considerada uma erva daninha sem importância econômica tem sido utilizada em várias populações com finalidade

medicinal e sua aplicação em diversas enfermidades pode ser devido a sua composição química, com efeitos inibitórios em microorganismos patogênicos além de uma forte atividade antioxidante (ABAJO et al., 2004).

Inúmeras atividades biológicas têm sido atribuídas a extratos vegetais obtidos a partir desta espécie, como por exemplo, inibidor da síntese de prostaglandinas (CHANG, C. L. et al., 2005; YANG et al., 2006), atividade antioxidante devido sua capacidade de não só suprimir a peroxidação lipídica como agir como seqüestrador de radicais livres e reduzir a depleção de GSH nessas células (YANG et al., 2006), atividade anti-inflamatória (PEREIRA et al., 1999) e ainda como agente capaz de aumentar a produção de citocinas Th2 como IL-10, interleucina anti-inflamatória, e de reduzir citocinas Th1 como TNF-α e INF-γ, mediadores pró-inflamatórios (CHANG, C. L. et al., 2005).

Diversos trabalhos demonstram o uso de extratos de *B.pilosa* no tratamento de lesões gástricas (ALVAREZ et al., 1999; HORIUCHI et al., 2010), diminuindo o estresse oxidativo no local da lesão, a produção de prostaglandinas e a inflamação presente (HORIUCHI et al., 2010). Além disso, trabalhos analisando sua atividade anti-inflamatória são abundantes. FOTSO et al. (2014), utilizando testes clássicos de dor e inflamação, demostraram a atividade analgésica e anti-inflamatória do extrato de *B.pilosa*, resultado similar ao encontrado por HORIUCHI; SEYAMA (2006).

PEREIRA et al. (1999) constataram que a atividade anti-inflamatória apresentada pelo extrato de *B.pilosa* pode ser devido a sua atividade imunossupressora, diminuindo a resposta proliferativa de linfócitos no local da lesão. Além disso, existem relatos de que o extrato dessa planta pode inibir a diferenciação de células T naive em células Th1 (CHIANG et al., 2007). Associado ao seu efeito anti-

inflamatório, *B.pilosa* apresenta um efeito antioxidante pronunciado, diminuindo a atividade da Superoxido dismutase (SOD), espécies reativas de oxigênio e a depleção de GSH citosólica (ABAJO et al., 2004; KOHDAA et al., 2013; YANG et al., 2006).

A seleção do extrato padronizado em ácido eicosapentanóico e linolênico a partir da espécie de *Bidens pilosa* L. (picão preto) se justifica ainda pelo papel dos ácidos graxos poli-insaturados como agentes anti-inflamatórios agindo na inibição da produção e liberação de mediadores pró-inflamatórios como eicosanóides (leucotrieno B_4, tromboxano A_2 e prostaglandina E_2) e citocinas (BELLUZZI et al., 2000; CAMUESCO et al., 2005; GIL, 2002), assim como por regularem a atividade de diferentes enzimas envolvidas no processo inflamatório, como óxido nítrico sintase induzível e ciclooxigenase (CAMUESCO et al., 2005; LEE, J. Y. et al., 2003).

Desse modo, o presente trabalho avaliou os efeitos do extrato de *Bidens pilosa* L. no modelo de inflamação intestinal induzida por TNBS, além de tentar elucidar quais mediadores participam dessa resposta. Foram avaliados mediadores inflamatórios relacionados à inflamação intestinal assim como mecanismos protetores da mucosa colônica.

Capítulo 1

Effects of *Bidens pilosa* L. extract in intestinal inflammation

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Key words: *Bidens pilosa,* colitis, cytokines, inflammation, inflammatory bowel disease, trinitrobenzenesulfonic acid.

Abstract

Bidens pilosa L. (Asteraceae), is a herb widely distributed that occurs in almost all tropical and subtropical countries and have been used in traditional medicine for its anti-inflammatory properties. Among other constituents, *B. pilosa* contain polyunsaturated fatty acids, that show anti-inflammatory and antioxidant properties, which are interesting for the treatment of inflammatory bowel disease (IBD). The aim of this study was to evaluate the intestinal anti-inflammatory effect of CO_2 supercritical extract of *B.pilosa* in the trinitrobenzenesulphonic acid (TNBS) model of intestinal inflammation. For this purpose, macroscopic parameters of lesion, biochemical markers (myeloperoxidase, alkaline phosphatase and glutathione) cytokines levels (IL-1β, IL-6, IL-10, TNF-α and INF-γ), and histologic evaluations (optic microscopy) were performed in rats with intestinal inflammation induced by trinitrobenzenesulphonic (TNBS) acid both in preventive and in curative protocols. In the preventive protocol *B.pilosa* at the dose of 25mg/kg was capable to diminish MPO and IL-1β levels. The dose of 50mg/kg decrease MPO and IL-1β levels associated with an increase in IL-10 levels and the 100mg/kg dose decrease extension of the lesion, MPO, IL-1β and TNF-α and prevent GSH depletion. In the curative protocol, the dose of 25mg/kg only decrease IL-1β; the dose of 50mg/kg was capable to diminish IL-1β, IL-6, IL-10 and TNF-α associated with a minor GSH depletion; the 100mg/kg dose was capable to decrease IL-1β, IL-10 and TNF-α. The extract of *B.pilosa* showed intestinal anti-inflammatory activity in the TNBS model of rat colitis confirming their traditional use in digestive inflammatory disease.

Introduction

Bidens pilosa L. (Asteraceae), is a herb widely distributed and occurring in almost all tropical and subtropical countries (ABAJO et al., 2004; CHANG, C. L. et al., 2005; KVIECINSKI et al., 2008; YANG et al., 2006). Although considered a weed without economic importance, *B.pilosa* has been used in various populations with medical purpose (ABAJO et al., 2004). Numerous biological activities have been attributed to plant extracts from this species, such as inhibitor of prostaglandin synthesis (CHANG, C. L. et al., 2005; YANG et al., 2006), antioxidant (YANG et al., 2006) and anti-inflammatory activities (PEREIRA et al., 1999) as well as an agent capable of increasing the production of IL-10 and reducing TNF-α (CHANG, C. L. et al., 2005).

Inflammatory bowel disease (IBD) is the common denomination of ulcerative colitis (UC) and Crohn's disease (CD). The etiology is unknown and the pathogenesis is complex and incompletely understood (BRENNA et al., 2013). Ulcerative colitis is a relapsing non-transmural inflammatory disease that is restricted to the colon; patients typically present with bloody diarrhoea, passage of pus, mucus, or both, and abdominal cramping during bowel movements (BAUMGART; SANDBORN, 2007). Crohn's disease is a relapsing, transmural inflammatory disease of the gastrointestinal mucosa that can affect the entire gastrointestinal tract from the mouth to the anus. The clinical presentation is largely dependent on disease location and can include diarrhoea, abdominal pain, fever, clinical signs of bowel obstruction, as well as passage of blood or mucus or both (BAUMGART; SANDBORN, 2007).

Medical treatment of Crohn's disease (CD) and ulcerative colitis (UC) includes a variety of drugs: aminosalicylates, corticosteroids, antibiotics, immunosuppressors, and

biological agents (anti-TNF) (SCRIBANO, 2008). However, none of them is specific to the disease and there is no cure for IBD (BREITRUCK et al., 2013; FIOCCHI, 2012).

Experimentally induced colitis with trinitrobenzene sulphonic acid (TNBS) is used to generate models that are used to examine the pathogenesis of gut inflammation, and determine the mechanisms and efficacy of therapies (JURJUS; KHOURY; REIMUND, 2004).

In this paper, we have study the anti-inflammatory activity of *B. pilosa* supercritical CO_2 fluid extract before and after TNBS instillation. Besides that we analyze several mediators that are related to intestinal inflammation and its alterations after extract administration.

Materials and methods

Reagents and plant material

All chemicals were provided by Sigma and Life Technologies. *B. pilosa* L. was cultivated using organic agricultural methods as certified by Ecocert Brazil (Santa Rosa de Lima, Santa Catarina, Brazil) and provided by Chemyunion Company Ltda. Taxonomic identification at Herbarium Irina Gemtchujnikov (Department of Botany, Institute of Biosciences, Universidade Estadual Paulista, UNESP, Botucatu, SP), where a voucher specimen was deposited. The aerial parts of *B.pilosa* was subjected to supercritical CO_2 fluid extraction and was standardized in phytol (0.139% (w/w)) and fatty acids (palmitic 30%, oleic 27%, linoleic 24,3% and linolenic 3,8%). This extract is commercially called Revinage ®. The substances were dissolved in 8% of tween 80 in methylcellulose (1% w/v) and prepared fresh daily for administration to the animals.

Animals

Male Wistar rats (weighing 180–200 g) obtained from the ANILAB – Animais de Laboratório, Paulínia, São Paulo (Brazil), were housed in standard environmental conditions (21ºC ± 2 ºC, 60–70% humidity) with 12 h light/dark cycles and air filtration. Animals had free access to water and food (Biobase). Experimental protocols met the ‘‘Guidelines of Animal Experimentation’’ approved by the Ethics Committee for Animal Research (Protocol number 042/04-CEEA), Institute of Biosciences, Unesp - Univ. Estadual Paulista.

Induction of colitis and assessment of the inflammatory process

Colitis was induced using the method originally described by MORRIS et al. (1989). Briefly, animals were fasted overnight and then anaesthetized. Under anesthesia, they were administered 10 mg of trinitrobenzenesulphonic acid (TNBS) dissolved in 0.25 ml 50% ethanol (v/v) using a Teflon cannula inserted 8 cm into the anus. During and after TNBS administration, the rats remained in a head-down position until they recovered from the anesthesia. Rats from the non-colitic (normal) group received 0.25 ml of saline instead of TNBS. After the colonic segments were obtained by laparotomy and the eventual occurrence of adhesions between the colon and adjacent organs were noted. The colons were weighed and its length measured under a constant load (2 g), then opened longitudinally and scored for macroscopically visible damage on a 0–10 scale by one observer unaware of the treatment, according to the criteria previously described (BELL; GALL; WALLACE, 1995): (no damage: 0; no ulcer, hyperemia: 1; linear ulcer with no significant inflammation: 2; linear ulcer with inflammation at one

site: 3; ≥ 2 sites of ulceration/inflammation: 4; ≥ 2major sites of ulceration and inflammation or one site of ulceration/inflammation extending 1 cm along the length of the colon: 5; if damage covers 2 cm along the length of the colon, the score is increased by 1 for each additional centimeter of involvement: 6 to 10). The colon was subsequently divided longitudinally into different pieces to be used for the biochemical determinations: myeloperoxidase (MPO) and alkaline phosphatase (ALP) activities and total glutathione (GSH) content, and cytokine evaluation (IL1-β, TNF-α, IL-6, INF-γ and IL-10).

Preventive treatment

Rats received 25, 50 or 100mg/kg of *B. pilosa* extract orally at 96, 72, 48, 24 and 2 hrs before colitis induction. The extract was administered using an esophageal catheter (volume: 10 ml/kg). Two additional groups were included for reference: a non-colitic group that received saline intracolonically and the vehicle orally, and a colitic group that received TNBS and vehicle (10 ml/kg methylcellulose + tween80) orally. The animal body weights, the occurrence of diarrhea (as detected by perianal fur soiling) and the total food intakes for each group were recorded daily. Animals from all groups (n =7) were killed 48 h after colitis induction.

Curative treatment

The extract was evaluated according to the experimental procedure of curative intestinal inflammation as described by DI STASI et al. (2004) with some modifications. In this protocol, colonic inflammation was induced with 10 mg of TNBS in 50% ethanol, as described previously. Treatments started 2 h after the administration of TNBS and continued daily for 7 days. In the eighth day the animals were killed. The

animals were divided randomly into five groups and treated with the same doses used in preventive experiment. The non-colitic and TNBS-control groups were also similar.

Biochemical assays

MPO activity was measured according to the technique previously described by KRAWISZ; SHARON; STENSON (1984). Samples were suspended in 1 ml of 50 mM phosphate buffer incorporating 0.5% hexadecyltrimethylammonium bromide (pH 6.0) and minced with scissors for 15 s on an ice-cold plate. The resultant suspension was subsequently diluted to a final 1:20 w/v ratio, homogenised for 1 min with an automatic Heidolphhomogeniser, sonicated for 10 s and subjected to three freeze-thaw cycles. The homogenates were then centrifuged at 7000g and 4°C for 10 min, and the supernatants were assayed for MPO activity. The results are expressed as MPO units per g of wet tissue. Total GSH content was quantified with the recycling assay(ANDERSON, 1985). Samples were thawed, minced, diluted to a concentration of 1:20 (w/v) in ice-cold 5% (w/v) trichloroacetic acid and homogenized. The homogenates were centrifuged at 2000g for 10 min at 4ºC, the supernatants were collected and centrifuged at 9000g for 5min at 4ºC and these supernatants were used to quantify glutathione content. The results are expressed as nmol per g of wet tissue. ALP activity was measured spectrophotometrically using disodium nitrophenyl phosphate (5.5.mM) as the substrate in 50 mM glycine buffer containing 0.5 mM $MgCl_2$, pH 10.5(BESSEY; LOWRY; BROCK, 1946; SMITH; HARRIS; PETERS, 1984).

Colonic samples for the determinations of TNF-α, IL-1β, IL-6, IL-10 and INF-γ were weighed, homogenized, minced on an ice-cold plate and resuspended in a centrifugation tube containing 10 mM/L phosphate buffered saline pH 7.4 (1:5 w/v). The tubes were placed in a shaker submerged in a 37^{o}C water bath for 20 minutes and

then centrifuged at 9000 x *g* for 30 s at 4°C. The supernatants were frozen at -80°C until assayed. The TNF-α, IL-1β, IL-6, IL-10 and INF-γ levels were quantified by a DuoSet ELISA Kit to measure the concentration of the natural and recombinant rat enzyme according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, Minnesota, USA). The results were expressed as pg per ml.

Optic microscopy analysis

Representative whole gut specimens were taken from a region 2 cm above the inflamed region of colon, corresponding to the segment adjacent to the gross macroscopic damage and were fixed in 4% buffered formaldehyde. Cross-sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the non colitic group. Full-thickness sections of 6 μm were obtained at different levels and stained with haematoxylin and eosin. After staining, images were subjected to analysis and photomicrography with a Leica microscope utilizing Leica Qwin Plus version 3.3 e 3.40.

Statistical Analysis

Parametric data are expressed as the mean ± S.E.M., and the differences between means were tested for statistical significance using one-way analysis of variance (ANOVA). Nonparametric data (score) are expressed as the median (range) and were analyzed with the Kruskal–Wallis test. Statistical significance was set at $p \leq 0.05$.

Results

Macroscopic evaluation

In preventive treatment intracolonic administration of TNBS resulted in an inflammation after 48h with a severe necrosis of the colonic mucosa, extending 1.8-3.9 cm along the colon, bowel wall thickening enhancing weight/length ratio, hyperaemia and adhesions to adjacent organs (table 1). This inflammatory process was associated with a reduction in food intake (data not shown) when compared with non-colitic animals and in consequence, a significant reduction in body weight was observed in TNBS-control group (data not shown). *B. pilosa* treatment shown a preventive effect at the dose of 100mg/kg being capable to reduce the extension of the lesion, the other doses had no effects on these parameters (table1).

In the curative protocol, the inflammatory process induced by intracolonic instillation of 10 mg of TNBS in 50% ethanol (v/v) progressed over time with characteristics similar to those previously reported (DI STASI et al., 2004; LUCHINI et al., 2008). Indeed, an alteration of the colonic absorptive function was noted since 50% of rats showed signs of diarrhea (data not showed). The colonic segments still appeared macroscopically ulcerated and inflamed, with increased colonic weight/length ratio and adhesions to adjacent organs. Only the dose of 50mg/kg of *B.pilosa* shows any improvement by reducing the extension of the lesion (Table 1).

Biochemical findings

In preventive protocol, colon damage was characterised biochemically by a 16-fold increase in MPO activity, a 3-fold increase in ALP activity and significant glutathione depletion in colon tissues (Table 2). In addition, colonic inflammation was also characterised by a significant increase in the levels of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and the anti-inflammatory cytokine IL-10 (figure1). *B. pilosa* showed a preventive effect at the dose of 100mg/kg demonstrated by lower MPO

activity, maintenance of glutathione level and reduction in IL-1β and TNF-α levels. Besides that the dose of 50mg/kg was capable to diminish MPO and IL-1β levels and enhance IL-10, an anti-inflammatory cytokine, level. The dose of 25mg/kg was capable to prevent MPO and IL-1β enhance. None of the doses used was capable to alter ALP, IL-6 and INF-γ levels (Table2 and Figure1).

On the other hand, in curative protocol, the administration of 50mg/kg of *B.pilosa* extract was capable to prevent GSH depletion and reduce IL-1β, IL-6 and IL-10 levels compared to TNBS-control group. The dose of 100mg/kg also prevents GSH depletion and diminishes IL-10 and TNF-α level. The dose of 25mg/kg only alters IL-1β level (Table 2 and Figure1).

Microscopic findings

Microscopic studies confirmed the beneficial effects of *B.pilosa* extract in all doses on TNBS-induced inflammation in rats. Colitic rats treated with these doses had an improved intestinal cytoarchitecture characterised by a restoration of the epithelial cell layer, presence of goblet cells, mucin replenishment, reduced edema in the submucosa layer and reduced inflammatory cell infiltration compared to TNBS control animals (Figure 2 and figure 3).

Discussion

The results of this study show for the first time that *B. pilosa* L. extract can be effective in the prevention and in treatment of the intestinal damage caused by TNBS administration, indicating the potential of this plant as a source of compounds with anti-inflammatory activity.

Oxidative stress results from an imbalance between the production of reactive species and the body's ability to manage them using endogenous antioxidants (DA COSTA; BADAWI; EL-SOHEMY, 2012). Reactive oxygen species exert deleterious effects by damaging cell structures and molecules (enzymes, proteins and DNA), leading to several gastrointestinal diseases, including inflammatory bowel disease (KIM, Y. J.; KIM; HAHM, 2012). Oxidative stress can be reduced by exogenous antioxidants, such as GSH, catalase, superoxide dismutase and others, or by the reduction in endogenous oxidants, such as MPO, ALP and nitric oxide.

MPO is a biochemical marker of neutrophil infiltration, and measurements of its activity have widely been used to detect intestinal inflammation (YAMADA et al., 1992). After TNBS administration occurs an enhance in MPO activity that can be associated with an enhance in neutrophil infiltration. The administration of *B. pilosa* extract at all doses diminishes this inflammatory marker in the preventive protocol, but none of the doses used were capable to alter this parameter in the curative protocol. According to KRAWISZ et al. (1984) and WINTERBOURN; BRENNAN (1997), compounds capable of decreasing the activity of MPO have potentially anti-inflammatory effects.

Associated with this enhance in MPO activity there is a depletion in GSH levels. Gluthatione is the first line of defense against the toxic products of oxygen, reducing hydrogen peroxide, lipid peroxides, and other hydroperoxides (DELEVE; KAPLOWITZ, 1991; MASELLA et al., 2005). Our results show depletion in GSH levels both in preventive and in curative protocols. In the preventive protocol only the higher dose of *B. pilosa* extract was capable to prevent this depletion. However, in the curative protocol the doses of 50 and 100mg/kg were capable to diminish the oxidative stress caused by TNBS administration. According LOGUERCIO et al. (2003),

glutathione supplementation decreases colonic damage by promoting the restoration of GSH and cysteine levels and therefore decrease lipid peroxidation in TNBS model of intestinal inflammation, results similar to which were found after *B. pilosa* treatment.

Cytokines have a crucial role in the pathogenesis of IBD, where they control multiple aspects of the inflammatory response. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines that occurs in IBD prevent the resolution of inflammation and instead leads to disease perpetuation and tissue destruction (NEURATH, 2014a). Cytokines are small glycoproteins produced by a number of cell types, predominantly leukocytes, which regulate immunity, inflammation and hematopoiesis. They regulate a number of physiological and pathological functions including innate immunity, acquired immunity and innumerous inflammatory responses (DINARELLO, 2011; KHAN, 2008). Dysregulation of these cytokines can lead to excessive inflammation or increased pathology, so, an alternative to control inflammatory diseases is to neutralize pro-inflammatory cytokines (DINARELLO, 2011). There are several studies that demonstrated the participation of cytokines in the IBD and in the TNBS model of intestinal inflammation (CORRIDONI; ARSENEAU; COMINELLI, 2014; HE et al., 2012; SANCHEZ-MUÑOZ; DOMINGUEZ-LOPEZ; YAMAMOTO-FURUSHO, 2008; XIONG et al., 2013). Among the most important cytokines are IL-1β, IL-6, IL-10 (an anti-inflammatory cytokine), TNF-α and INF-γ.

Lymphocytes and antigen presenting cells orchestrate a lot of the inflammation in IBD, mainly the production of TNF-α, that exerts its pro-inflammatory effects through increased production of IL-1β and IL-6 (SANCHEZ-MUÑOZ et al., 2008). In IBD, TNF-α function extend beyond pro-inflammatory properties. TNF-α can activate endothelial cells, induce chemokines, recruit neutrophils to the inflamed gut mucosa,

induce edema, activate coagulation and participate in granuloma formation (VAN DEVENTER, 1997). There was an increase in TNF-α level after TNBS instillation both in preventive and in curative protocols. The administration of *B.pilosa* in the dose of 100mg/kg was capable to diminish this cytokine level in both protocols indicating that this extract can act as a potential immunomodulator.

Associated with elevated levels of TNF-α were found enhanced IL-1β. According to COCCIA et al. (2012) IL-1β in the colon is correlated with disease activity and high levels of IL-1β were associated with active lesions, suggesting an important role of this cytokine in promoting localized inflammation. IL-1β, similar to TNF-α is a multifunctional cytokine with biological activities that are of particular relevance to IBD, for example, neutrophil recruitment and accumulation, immune cell activation, endothelial adhesion molecule expression, acute-phase protein synthesis, and the production of eicosanoids, which participate in the tissue destruction in IBD (DIONNE et al., 1997). In the curative protocol, the doses of 25 and 50mg/kg were capable to diminish this cytokine level; however in the preventive protocol all doses were capable to do that. These results can be interpreted as an anti-inflammatory activity of the extract once several studies demonstrated that the treatment with IL-1β -blocking agents has been successful in ameliorating acute models of intestinal injury and inflammation (COMINELLI et al., 1992; SIEGMUND et al., 2001; THOMAS et al., 1991).

The third pro-inflammatory cytokine analyzed was IL-6. Although mostly regarded as a pro-inflammatory cytokine, IL-6 also has many regenerative or anti-inflammatory activities (SCHELLER et al., 2011). Among the cytokines, IL-6 has a positive correlation with the disease activities of IBD, and its production returns to normal levels when gut inflammation becomes inactive (LEE, M. J. et al., 2012). IL-6

plays an important role in enhancing T-cell survival and it is also involved in the immune deviation of regulatory T cells toward inflammatory cells (e.g., Th17) (BETTELLI et al., 2006). Our results show that IL-6 was elevated both on preventive and in curative protocols indicating a participation of this cytokine in the TNBS model of inflammation. In the curative model, the administration of 50mg/kg of *B. pilosa* extract was capable to diminish IL-6 level as expected, since there is a lower inflammation characterized by minor extension of the lesion; on the other hand, in the preventive model, none of the doses utilized alter this parameter.

XING et al. (1998), in an elegant study, demonstrate that IL-6 is critically required to control the extent of systemic acute inflammatory responses, particularly the level of pro inflammatory cytokines in the local of inflammation. Besides that, in a lung inflammation, the absence of IL-6 not only resulted in a more pronounced response of proinflammatory cytokines but a greater extent of tissue neutrophilia, suggesting that in a normal host, one function of inducible IL-6 during acute responses is to suppress the level of proinflammatory cytokines without compromising the level of anti-inflammatory cytokines (XING et al., 1998). In this way, the maintenance of IL-6 levels during the preventive protocol, considered an acute form of inflammation, can be interpreted as a beneficial step in the inflammatory resolution control caused by the administration of *Bidens* extract. This result can be associated with an increase in IL-10 levels, a widely known immunosuppressive cytokine by its ability to inhibit macrophage-dependent antigen presentation, T-cell proliferation, and Th1 cytokine secretion (SANTIN et al., 2000).

In our experiments there is an IL-10 level enhance in TNBS-control group both in preventive and in curative protocols, supporting the premise that these IL-10 levels may be protecting the epithelial cells surface from injury. In the preventive protocol,

there is also an enhance after *Bidens* treatment in dose of 50mg/kg, this enhance can be considered beneficial, protecting the tissue from the TNBS damage. These results are similar to what BARBARA et al. (2000) found with gene therapy using an adenovirus-IL-10 in TNBS model of intestinal inflammation. In this study IL-10 successfully ameliorated colonic inflammation and tissue injury when the adeno-IL-10 construct was transferred before the induction of colitis. When the adeno-IL-10 construct was administered after induction of the colitis had little or no effect. This results indicates that enhance in IL-10 level is beneficial if it occurs prior to inflammation, being more prominent in the prevention of relapses than in the suppression of established inflammation. This is similar to what occurs in the curative protocol, where the TNBS is administered prior to the treatment. In this protocol, *B. pilosa* extract diminished IL-10 levels at dose of 50 and 100mg/kg. This result associated with lower IL-1β, IL-6 and TNFα levels indicates a minor inflammation in the site of TNBS instillation.

All these results together indicate a potential use of *B. pilosa* extract in the prevention and/or amelioration of intestinal inflammation. Probably the anti-inflammatory effects presented here is duo to inhibition of pro-inflammatory cytokines, minor neutrophil infiltration demonstrated by lower MPO activity and anti-oxidant properties verified by maintenance of GSH levels. Taken together, the data indicate a potent immunosuppressive action of *B. pilosa* extract, suggesting a promising application as an anti-inflammatory drug.

Conflict of interest statement

The authors declare that there is no conflict of interest

Acknowledgement

Research in the Di Stasi lab (PhytoPharmaTech) is supported by São Paulo Research Foundation (FAPESP), grant number 11/50512-2. Fellowships: AEVQ from National Council for Scientific and Technological Development (CNPq - Brazilian Ministry of Science and Technology), LDAJ from Coordination for the Improvement of Higher Education Personnel (CAPES-Brazilian Ministry of Education), CARAC from São Paulo Research Foundation (FAPESP) and LCDS from National Council for Scientific and Technological Development (CNPq - Brazilian Ministry of Science and Technology). The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of financial support agencies.

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Figure Legends

Figure 1

Effects of *Bidens pilosa* L. extract in doses of 25mg/kg, 50mg/kg or 100mg/kg on the levels of IL-1β, IL-6, IL-10 and TNF-α in colon from rats with TNBS-induced intestinal inflammation.

Figure 2

Colonic sample photomicrographs from preventive protocol. In A (non-colitic group): the mucosa (m) contains numerous straight tubular glands (white arrows) with many lightly stained goblet cells; the normal crypt (black arrow), submucosa (sm) and luminal epithelium was intact with a typical morphology. In B (TNBS group): tubular glands (white arrows) were reduced and the goblet cells were atypical; a disruption of the epithelium (black arrow) with extensive ulcerations and a notable oedema (oe) containing many inflammatory cells can be observed. In C (25mg/kg treated group), D (50mg/kg treated group) and E (100mg/kg treated group): colon cytoarchitecture was recovering and included the restoration of tubular glands (white arrows) containing goblet cells, which were similar to healthy animals; oedema (oe) were reduced; the epithelium was intact and inflammatory cells were completely reduced.

Figure 3

Colonic sample photomicrographs from curative protocol. In A (non-colitic group): the mucosa (m) contains numerous straight tubular glands (white arrows) with many lightly stained goblet cells; submucosa (sm) and luminal epithelium was intact with a typical morphology. There is no signal of oedema, as expected. In B (TNBS group): tubular glands (white arrows) were reduced and the goblet cells were atypical; a disruption of the epithelium (black arrow) with extensive ulcerations and a notable oedema (oe)

containing many inflammatory cells can be observed. In C (25mg/kg treated group), D (50mg/kg treated group): colon cytoarchitecture was recovering and included reduced oedema (oe) and inflammatory cells; the epithelium was intact and occurs a restoration of tubular glands (white arrows) containing goblet cells, which were similar to healthy animals. In E (100mg/kg treated group) there is minor oedema (oe) and inflammatory cells; the is minor restoration of the tubular glands compared to the others two doses (white arrows).

Figure 1

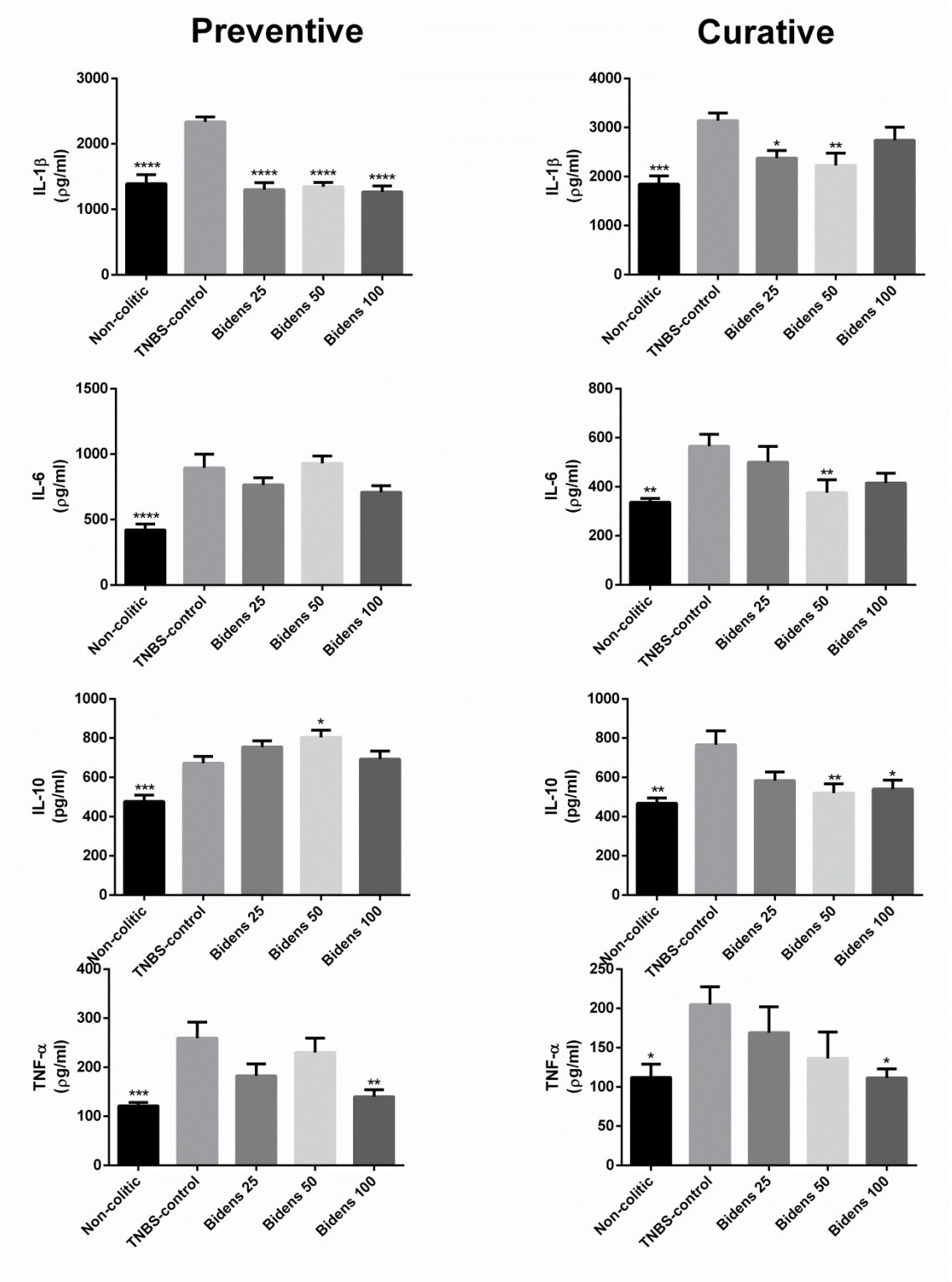
Preventive
Curative
IL-1β (ρg/ml)
IL-6 (ρg/ml)
IL-10 (pg/ml)
TNF-α (ρg/ml)
Non-colitic
TNBS-control
Bidens 25
Bidens 50
Bidens 100

Figure 2

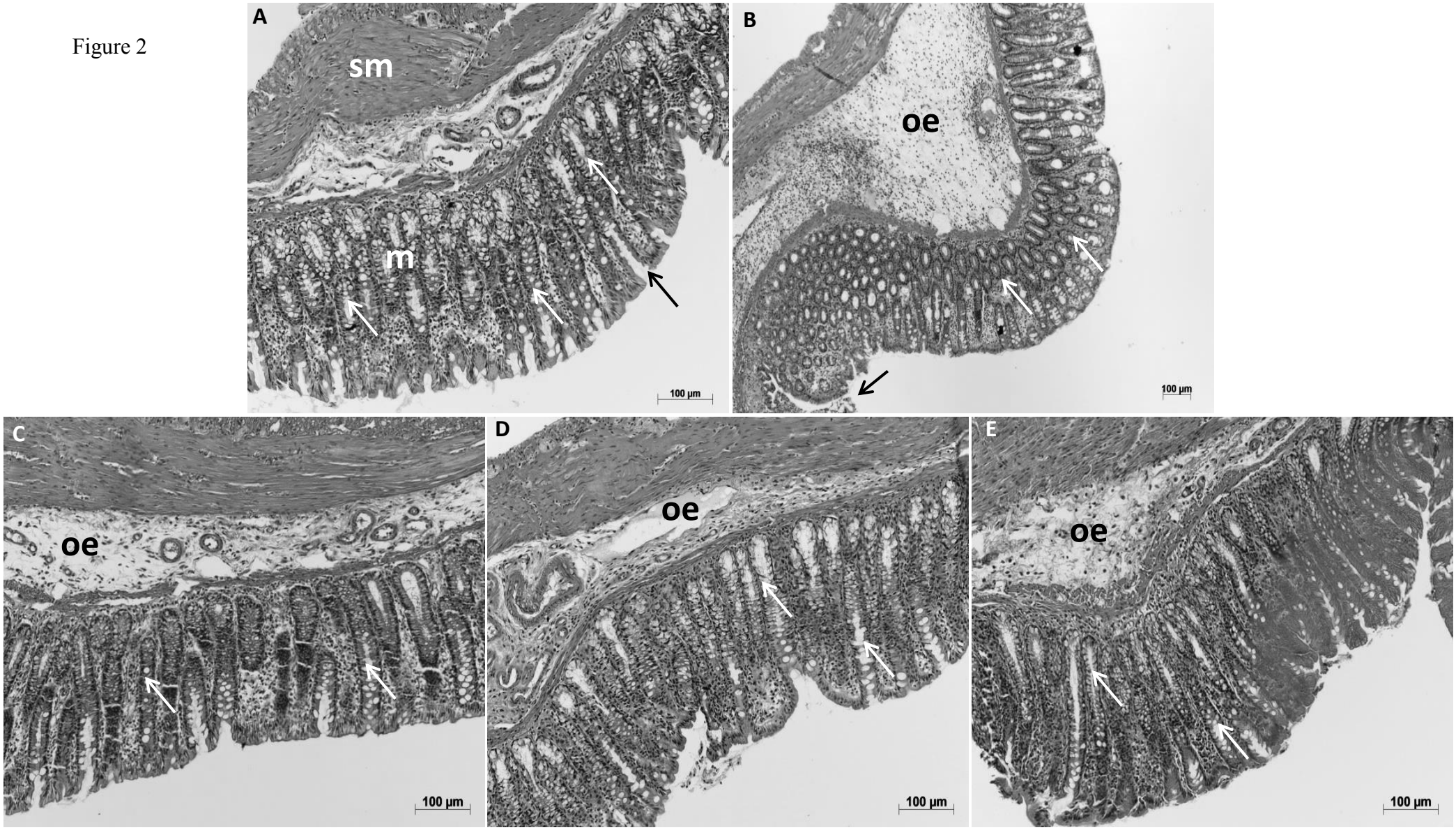

Figure 3

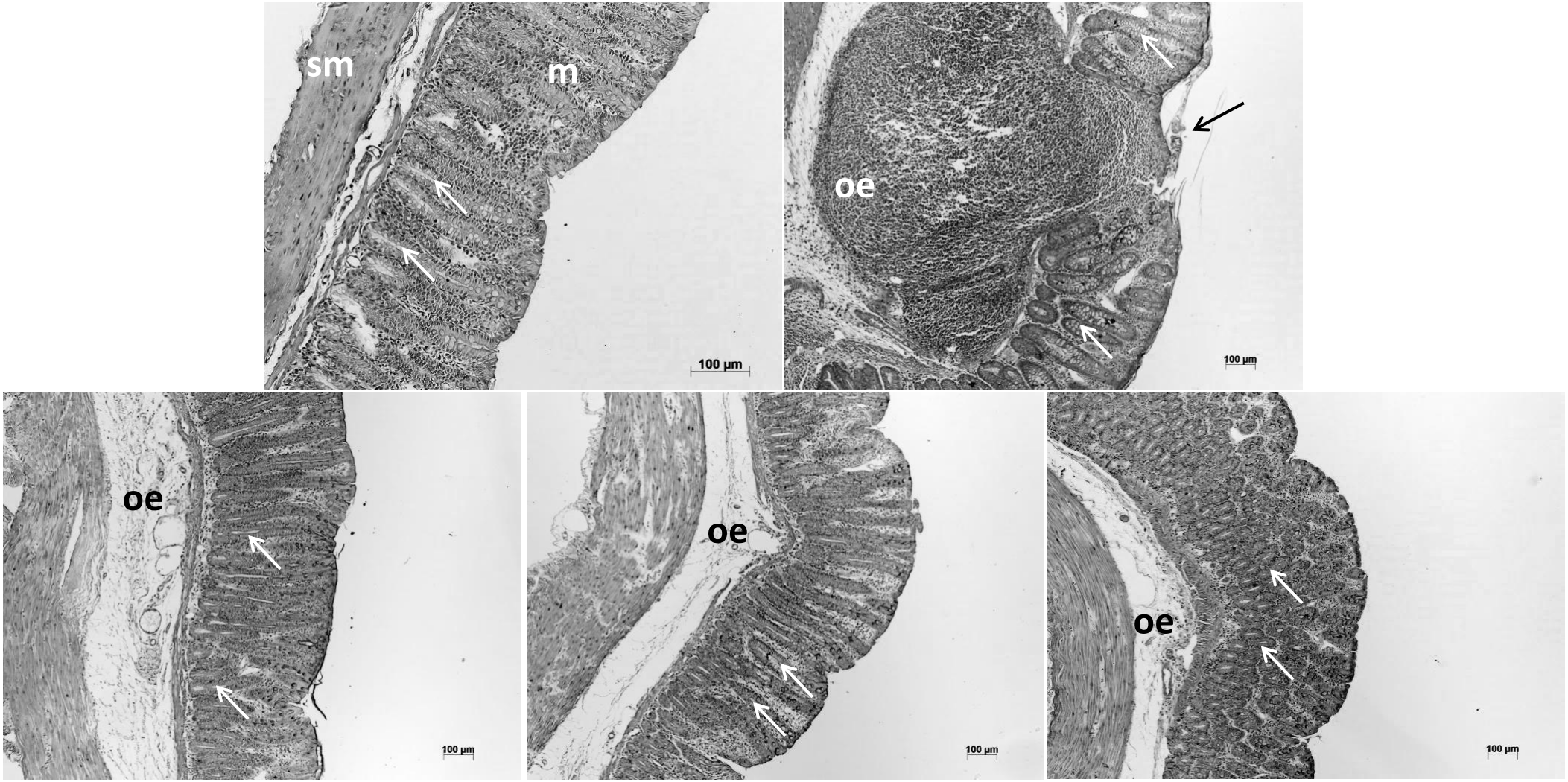

Table 1. Effects of *Bidens pilosa* L. treatment on macroscopic parameters in TNBS-induced intestinal inflammation

		Macroscopic damage score (0–10)[1]	Extension of lesion (cm)[2]	Weight/length ratio (mg/cm)[2]
Preventive	Non-colitic	0 (0-0)*	0.0±0.0*	82.7±2.1*
	TNBS-control	7.5 (5-8)	2.8±0.2	140.8± 4.5
	Bidens 25	6 (2-9)	2.2±0.6	157.9±5.6
	Bidens 50	8 (5-9)	2.8±0.2	135.6±6.4
	Bidens 100	5 (4-9)	1.6±0.6*	142.6±5.5
Curative	Non-colitic	0 (0-0)*	0.0±0.0*	90,0±2.0*
	TNBS-control	7 (6-8)	2.7±0.2	186.2±29.2
	Bidens 25	6 (5-10)	3.3±0.7	278.7±82.9
	Bidens 50	5 (1-7)	1.6±0.5*	191.3±36.2
	Bidens 100	6 (5-7)	2.2±0.3	151.8±7.6

[1]Score data are expressed as the median (range). [2] Extension of lesion and weight/length ratio are expressed as the mean ± S.E.M. *$p < 0.05$.

Table 2. Effects of *Bidens pilosa* L. treatment on biochemical parameters in TNBS-induced intestinal inflammation

		GSH	MPO	ALP
Preventive	Non-colitic	1687±54.2*	104±13.7****	8.91±2.2****
	TNBS-control	1295±60.1	1703±390.5	28.9±3.1
	Bidens 25	1565±116.0	613±294.6**	19.0±3.2
	Bidens 50	1316±60.2	624±121.5**	29.2±4.0
	Bidens 100	1973±217.0***	714±115.9*	21.0±2.3
Curative	Non-colitic	1753±94.1**	84±8.8***	5.7±0.4**
	TNBS-control	1255±82.4	831±145.5	13.9±1.5
	Bidens 25	1216±91.4	795±132.7	13.8±1.2
	Bidens 50	1699±70.6**	593±168.9	16.9±3.6
	Bidens 100	1798±112.0***	731±143.6	13.5±1.4

Data are expressed as the mean ± S.E.M. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001

Table 3. Effects of *Bidens pilosa* L. treatment on cytokine levels in TNBS-induced intestinal inflammation

		IL-1β	IL-6	IL-10	TNF-α	INF-γ
Preventive	Non-colitic	1395±136.9****	421±45.3****	477±32.2***	121±7.1***	84±9.2
	TNBS-control	2337±73.9	894±105.5	672±35.5	259±32.4	125±20.4
	Bidens 25	1301±107.2****	765±54.2	754±31.7	183±24.3	117±7.6
	Bidens 50	1346±66.5****	929±55.7	804±36.0*	230±28.9	91±10.4
	Bidens 100	1267±92.7****	709±49.6	693±40.6	140±14.4**	93±12.9
Curative	Non-colitic	1846±166.8***	337±15.5**	468±26.5**	112±17.0*	125±12.0
	TNBS-control	3139±156.0	566±47.7	766±71.2	205±22.7	113±8.2
	Bidens 25	2380±151.6*	501±64.6	584±43.4	169±32.8	133±17.4
	Bidens 50	2228±248.2**	376±52.0**	520±47.0**	136±33.5	98±8.4
	Bidens 100	2743±262.3	415±40.2	541±45.8*	112±11.4*	77±4.7

Data are expressed as the mean ± S.E.M. $^{*}p < 0.05$, **p<0.01, ***p<0.001, ****p<0.0001

Capítulo 2

Molecular and Ultra-structural changes caused by *Bidens* pilosa L. administration in TNBS-induced intestinal inflammation

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Key words: *Bidens pilosa,* colitis, HSP70, Heparanase, Mucins, inflammation, inflammatory bowel disease, trinitrobenzenesulfonic acid.

Abstract

Inflammatory Bowel Disease (IBD) comprises basically two distinct diseases: Crohn's disease (CD) and ulcerative colitis (UC), being characterized by a chronic inflammation of the intestine, with exacerbation of symptoms followed by periods of remission. There is no cure and the current pharmacological treatments present several side effects. Previously studies from our group found that *B.pilosa* extract is capable to diminish intestinal inflammation caused by TNBS, decreasing pro-inflammatory cytokines such as IL-1β and TNF-α. Based on that, we proposed to study the molecular and ultra-structural effects of *B. pilosa* supercritical CO_2 fluid extract before and after TNBS instillation in rats. In the preventive protocol we found that *B.pilosa* extract at the dose of 25mg/kg was capable to decrease HSP70, MAPK3 and MUC1 gene expression associated with enhanced expression of MUC3 and MUC4. The dose of 50mg/kg decrease Heparanase, MAPK3, MUC1 and MUC2 gene expression and the dose of 100mg/kg decrease HSP70, heparanase, MAPK3, MUC1 and MUC2 expression. In the curative protocol HSP70 and MAPK1 were decreased by all doses and the dose of 25mg/kg was capable to enhance MUC1 gene expression. The microscopic analysis shown that occurs an improvement in colonic appearance, indicating minor oedema and membrane disruption and a mucin production enhance in all doses analyzed. Altogether, these results indicate the use of *B.pilosa* extract as a possible source of useful compounds to treat human IBD.

Introduction

Inflammatory Bowel Disease (IBD) comprises basically two distinct diseases: Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are characterized by a chronic inflammation of the intestine, with exacerbation of symptoms followed by periods of remission (ARSENEAU et al., 2007; ROSENSTIEL et al., 2009; STROBER et al., 2007). Although there are some differences, neutrophil infiltration and ulceration on the mucosa, abdominal discomfort or pain with altered bowel habits such as diarrhea and constipation occur in both diseases (SINGH et al., 2003). UC is restricted to colonic mucosal layer and is characterized by infiltration of lymphocytes and granulocytes and presence of crypt abscesses with neutrophil infiltration (COLLINS; CROITORU, 1994), whereas CD is a transmural inflammation of the gastrointestinal tract that can affect discontinuously from the mouth to the anus and can promote complications such as stenosis, abscesses or fistulas. This disease is also characterized by infiltration of lymphocytes, granuloma formation and fibrosis (BAUMGART; SANDBORN, 2007; COLLINS; CROITORU, 1994).

Current therapies involve a combination of aminosalicylates, corticosteroids, immunosuppressants and recently monoclonal antibodies against tumour necrosis factor α (TNF-α) (GIRARDIN et al., 2012; PITHADIA; JAIN, 2011; YAMAMOTO-FURUSHO, 2007). These drugs have shown beneficial effects in the treatment of IBD, but present several side effects and not all patients respond to these treatments (YAMAMOTO-FURUSHO, 2007). Thereby, research of new agents that combine pharmacologic efficacy and the absence of undesirable adverse effects is critical.

Bidens pilosa L., Asteraceae family, is a weed with wide distribution and occurrence in countries in Africa, North America, Central and South America and Asia (ABAJO et al., 2004; CHANG, C. L. et al., 2005; HORIUCHI et al., 2010;

KVIECINSKI et al., 2008). Several studies demonstrate the use of *B.pilosa* extracts in the treatment of gastric lesions (ALVAREZ et al., 1999; HORIUCHI et al., 2010), reducing the oxidative stress at the site of injury and production of inflammatory mediators (HORIUCHI et al., 2010). In addition, FOTSO et al. (2014), using classical pain and inflammation tests, demonstrated the analgesic and anti-inflammatory activities of *B.pilosa* extract, results similar to were observed by HORIUCHI; SEYAMA (2006).

Previously studies from our group found that *B.pilosa* extract is capable to diminish intestinal inflammation caused by TNBS, decreasing pro-inflammatory cytokines such as IL-1β and TNF-α (Quaglio et al, 2015). Based on the previous results, we proposed to study molecular and ultra-structural effects of *B. pilosa* supercritical CO_2 fluid extract before and after TNBS instillation in rats. These studies will improve the understanding of how *B.pilosa* extract could be acting to decrease intestinal inflammation.

Materials and methods

Reagents and plant material

All chemicals were provided by Sigma and Life Technologies. *B. pilosa* L. was cultivated using organic agricultural methods as certified by Ecocert Brazil (Santa Rosa de Lima, Santa Catarina, Brazil) and provided by Chemyunion Company Ltda. Taxonomic identification at Herbarium Irina Gemtchujnikov (Department of Botany, Institute of Biosciences, Universidade Estadual Paulista, UNESP, Botucatu, SP), where a voucher specimen was deposited. The aerial parts of *B.pilosa* was subjected to

supercritical CO_2 fluid extraction and was standardized in phytol (0.139% (w/w)) and fatty acids (palmitic 30%, oleic 27%, linoleic 24,3% and linolenic 3,8%). This extract is commercially called Revinage ®. The substances were dissolved in 8% of tween 80 in methylcellulose (1% w/v) and prepared fresh daily for administration to the animals.

Animals

Male Wistar rats (weighing 180–200 g) obtained from the ANILAB – Animais de Laboratório, Paulínia, São Paulo (Brazil), were housed in standard environmental conditions (21ºC ± 2 ºC, 60–70% humidity) with 12 h light/dark cycles and air filtration. Animals had free access to water and food (Biobase). Experimental protocols met the ‘‘Guidelines of Animal Experimentation’’ approved by the Ethics Committee for Animal Research (Protocol number 042/04-CEEA), Institute of Biosciences, Unesp - Univ. Estadual Paulista.

Induction of colitis

Colitis was induced using the method originally described by MORRIS et al. (1989). The preventive and curative protocols were made based in our previously work (Quaglio et al, 2015). After that, the colon was divided for the gene expression analysis (β-actin, HPRT, GAPDH, HSP70, Heparanase, NF-κB, MAPK1, MAPK3, MAPK6, MAPK9, MUC1, MUC2, MUC3 e MUC4).

Gene expression analysis

Colon samples (100mg) were collected and stored in -80°C until use. For the homogenization, were used 1ml of Trizol® (Invitrogen™ Life Technologies) and a

Polytron. The total RNA extraction was made according to the Trizol® manufacturer's protocol. The purity was determined by A260/A280 ratio using a Nanodrop 2000 (Thermo Scientific). After that, 1 μg from total RNA of colon tissue samples were incubated with DNAse I (1 U/mg RNA; Invitrogen™), and then reverse transcribed with SuperScript® III (200 U/ml; Life Technologies™) and oligo-d (T) primer. Primers for targets and reference genes were designed based on the rat sequences using IDTprimerquest software (http://www.idtdna.com/primerquest/Home/Index; Invitrogen™). Relative real-time RT-PCR analysis was performed with an StepOne Plus™ (Applied Biosystems®) using Power SYBR Green PCR Master Mix® (Life Technologies™) for all the genes. Amplification efficiencies for target and reference genes were similar. The primer sequences, fragment size, annealing temperature, primer concentration, NCBI Reference Sequence and sample concentration for each gene are shown in Table 1.

Reactions were optimized to provide maximum amplification efficiency for each gene. PCR was performed in 25 μl reaction volumes in duplicate, in a MicroAmp® Fast Optical 96-Well Reaction Plate, 0.1 mL (Applied Biosystems®) and the specificity of each PCR product was determined by melting curve analysis. Negative controls (water replacing cDNA) were run in every plate.

The relative expression of each target gene was calculated using the DDCt method with efficiency correction (PFAFFL, 2001); the control was a cDNA sample from each cell type analyzed. To select the most stable reference gene for detailed analyses, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), β-actin and HPRT amplification profiles were compared using the RefFinder software (http://www.leonxie.com/referencegene.php?type=reference). All gene expression analysis were performed with β-actin as the reference gene for colon tissue

Sscanning electron (SEM) and transmission electron (TEM) microscopy analysis

For morphological analysis by scanning electron microscopy (SEM), colon samples from healthy, control and treated animals were fixed for 24h in 2.5% glutaraldehyde with 0.1M phosphate buffer, pH 7.3. In addition, solon samples were post-fixed with 1% osmium tetroxide for 2 h, dehydrated in a graded alcohol series, critical point dried, coated with gold and examined under a Fei-Quanta 200 scanning electron microscope (Phillips, Czechoslovakia).

PAS/Alcian blue stain

For PAS/Alcian Blue analysis was used the protocol previously described by LINDEN; FLORIN; MCGUCKIN (2008). De-waxed sections were immersed in 100% ethanol for 10 min, rinsed in water for 10 min, immersed in 3% acetic acid for 2 min and stained in 1% Alcian Blue 8GX in 3% acetic acid (pH 2.5) for 2.5 h. Nonspecific stain was removed with 3% acetic acid and rinsed in water for 10 min. The slides were then oxidized in 1% periodic acid in water at room temperature for 10 min, washed in water for 5 min, immersed in Schiff's reagent for 10 min, rinsed in water for 5 min and then three times in 0.5% sodium metabisulphite before a final wash in water. To reveal O-acetylated oligosaccharides sections were first treated with 0.1 M KOH for 30 min and then 1 mM periodic acid prior to the Schiff reagent.

Statistical Analysis

Parametric data are expressed as the mean ± S.E.M., and the differences between means were tested for statistical significance using one-way analysis of variance (ANOVA) and Dunnet's posteriori test. Statistical significance was set at $p \leq 0.05$.

Results

Gene expression findings

In the preventive protocol, occurs an enhance in HSP70, heparanase, MAPK3, MUC1 and MUC2 gene expression in colitic animals compared to healthy ones. The expression of MAPK1, MAPK6, MAPK9 and NF-κB were not altered after the TNBS instillation. The administration of *B. pilosa* extract at 25mg/kg was effective in reduce HSP70, MAPK3, MUC1 and MUC2 besides enhance MUC3 and MUC4 gene expression, two mucins that are not altered by TNBS alone. The administration of 50mg/kg was capable to reduce Heparanase, MAPK3, MUC1 and MUC2. Finally, the administration of 100mg/kg diminishes HSP70, Heparanase, MAPK3, MUC1 and MUC2 gene expression but.

In curative protocol, only HSP70, Heparanase and MUC2 are altered after TNBS administration. The administration of 25mg/kg of *B. pilosa* extract was effective in diminish HSP70 expression levels and enhance MUC1. The 50mg/kg dose diminishes HSP70, heparanase and MAPK1 expression and the 100mg/kg dose only alters HSP70 gene expression levels (Table 2 and 3).

Microscopy findings

In the Scanning Electron Microscopy (figure 1) evaluation several morphological changes were observed in preventive and curative protocols. Preventive protocol: In 1 (non-colitic group): a regular mucosal architecture with polygonal units as structural subunits, regular microvilli giving a smooth velvety appearance, crypts (white arrows), goblet cells (black arrows) and some mucin (m) extruded. In 2 (TNBS-control group): a complete loss of the smooth velvety appearance and polygonal shape

of absorptive cells and crypts (white arrows), hyperplasia of goblet cells (black arrows), mucin extrusion greater than in non-colitic group (m). In 3 (25mg/kg group): recovery of polygonal shape of absorptive cells and crypts although high number of goblet cells (black arrows) and lesser mucin extraction (m). In 4 (50mg/kg group): a minor number of goblet cells (black arrows), a recovery of polygonal appearance and increased mucin (m) compared to TNBS-control. In 5 (100mg/kg group): a good recovery of mucosa architecture and normal number of goblet cells, great mucin extraction covering almost all colon (m).

Some resemblances were observed in curative protocol: In 1 (non-colitic group): a regular mucosal architecture with crypts (white arrows), goblet cells (black arrows), regular microvilli giving a smooth velvety appearance and mucin (m) extruded. In 2 (TNBS-control group): a complete loss of the smooth velvety appearance and polygonal shape of absorptive cells, hyperplasia of goblet cells (black arrows), loss of mucin extrusion. In 3 (25mg/kg group): recovery of polygonal shape of absorptive cells and crypts and smaller number of goblet cells (black arrows) although high mucin extraction (m). In 4 (50mg/kg group): an intermediate number of goblet cells (black arrows), a recovery of polygonal appearance (white arrow) and increased mucin (m) compared to TNBS-control. In 5 (100mg/kg group): a good recovery of mucosa architecture although high number of goblet cells (black arrows) and increased mucin extraction compared to TNBS-control group (m).

In the Transmission Electron Microscopy evaluation several morphological changes were observed in preventive (figure 1) and curative protocols. Transmission electron microscopy - Preventive protocol: In 1 (non-colitic group): the cells are anchored to the underlying basement membrane complex; absorptive epithelial cells with a well-developed brush border containing numerous regular microvilli (black

arrows) typical of enterocytes and its nucleus (n) with chromatin in a basal position (white arrow). Colonic goblet cells (G) showing the mucus granules (m). In 2 (TNBS-control group): mucin granules in colonic goblet cells are reduced; surface cells have atypical microvilli (black arrow) and intercellular space was enhanced as a signal of edema (white arrows). In 3 (25mg/kg group): the goblet cells (G) showing the mucus granules (m) are more similar to healthy animals that to colitic ones with nucleus in basal position; normal intercellular space with a few signals of oedema and surface cells have atypical microvilli (black arrow). In 4 (50mg/kg group) and 5 (100mg/kg): surface cells with a brush border and preserved typical microvilli (black arrows), goblet cells (G) containing mucus granules (m) similar to healthy animals with nucleus in basal position and typical cellular space.

Curative protocol: In 1 (non-colitic group): absorptive epithelial cells with a well-developed brush border containing numerous regular microvilli (black arrows) typical of enterocytes and its nucleus (n), with chromatin, in a basal position (white arrow). Colonic goblet cells (G) showing the mucus granules (m). In 2 (TNBS-control group): mucin granules in colonic goblet cells are reduced and disorganized; surface cells have atypical microvilli (black arrow) and intercellular space was enhanced as a signal of edema (white arrows). In 3 (25mg/kg group), 4 (50mg/kg) and 5 (100mg/kg): the goblet cells (G) showing the mucus granules (m) similar to healthy animals with nucleus in basal position; normal intercellular space and surface cells with a brush border and preserved typical microvilli (black arrows).

Alcian Blue/PAS findings

In the preventive protocol (figure 2), the non-colitic group (1) presents as expected, a smooth blue (white arrows) staining in the mucosa (m), indicating the

presence of mucin in the goblet cells. There is also a thin blue layer in the membrane, representing the membrane-bond mucin (black arrow). In TNBS-control group (2), there was mucin depletion both in the mucosa (m) and cell surface (black arrow) associated with a minor blue staining. Associated with that can be notice a large oedema (oe) between mucosa (m) and submucosa (sm). In 3 (25mg/kg group), 4 (50mg/kg group) and 5 (100mg/kg group) the goblet cells in the mucosa (m) are replenish with mucin (white arrows) associated with higher blue staining. There is no oedema and a thin blue layer indicating the membrane-bond mucin (black arrows).

In curative protocol (figure 2), the non-colitic group (1) showed a mild blue staining, indicating the presence of mucin both in mucosa (m) and cell surface (black arrows). The goblet cells are filled with mucin (white arrows). In 2 (TNBS-control group) the goblet cells are damage and disarranged with a smooth blue staining (black arrows). There is no blue layer in the cell surface. It can be notice also the presence of an oedema between mucosa (m) and submucosa. In 3 (25mg/kg) and 4 (50mg/kg) there is a stronger blue staining in the mucosa (m) (white arrows) compared to TNBS-control group, besides that there is a thick blue layer in the cell surface representing the membrane-bond mucin (black arrows) associated with a minor oedema (oe) in the mucosa. In 5 (100mg/kg) the goblet cells are replenished with mucin (white arrows), there is no oedema and a thin layer in the cell surface (black arrows).

Discussion

Currently, the pharmacological treatment of IBD is mainly based on the administration of anti-inflammatory agents, immunosuppressants or biological agents. However, and in many cases, these drugs presents relevant side effects, especially with long-term use and a lot of patients are refractory to these conventional treatments. This

has led to an increasing interest in the search for new compounds that can be use as complementary medicine in order to obtain clinical efficacy with minimal side effects (ALGIERI et al., 2013). In this study, we showed that *Bidens pilosa* L. can be a good source of products to treat IBD.

In previous studies, we found that *B.pilosa* extract was effective in diminishes several pro-inflammatory mediators; besides enhance anti-inflammatory ones both in preventive and in curative models of intestinal inflammation in rats. Thereby, the present study aimed to investigate the molecular and ultrastructural mechanisms that lie behind the effects produced by this extract.

We observed that *B. pilosa* was able to prevent HSP70 enhance induced by TNBS instillation. This effect was observed after treatment with 25 and 100 mg/Kg in preventive protocol and with all doses in curative protocol. HSP70 belongs to a family of molecules highly conserved throughout evolution that are typically involved in folding, refolding, translocation and degradation of intracellular proteins under normal and stress conditions (TOMASELLO et al., 2011). HSP70 is quickly synthetized after exposure to different stressors to maintain homeostasis, repair damaged areas and provide protection against injuries (KAUFMANN, 1990; LIU et al., 2010). As expected, there is greater HSP70 gene expression after TNBS instillation due to a major stress in the colon both in preventive and in curative protocols. This damage is similar to what occurs in IBD patients and in DSS (Dextran sulfate sodium) model of intestinal inflammation (CHEN; NOBLE, 2009; DUDEJA; VICKERS; SALUJA, 2009; LUDWIG et al., 1999; O PETROF; CIANCIO; CHANG, 2004; TANAKA et al., 2007). In the preventive protocol, the reduced HSP70 expression indicates that *B. pilosa* protected against TNBS instillation damage, whereas in the curative treatment, it is possible that extract was able to decrease local inflammation and consequent local

stress. These results can be confirmed by the microscopic findings, in which all doses of *B. pilosa* extract diminish the oedema associated with a preservation of the mucosa and goblet cells.

Heparanase is an endo-β-D-glucuronidase capable of cleaving heparan sulfate (HS) side chains releasing multitude HS-bound biological mediators such as growth factors, cytokines and chemokines (BEN-ZAKEN et al., 2008; DOVINER et al., 2006; MCKENZIE, 2007). Heparanase is the only functional endoglycosidase capable of cleaving heparan sulfate (HS) in mammals, activity implicated in cell dissemination associated with tumor metastasis and inflammation (SHTEINGAUZ; ILAN; VLODAVSKY, 2014). In the TNBS-control group occurs an enhancement in this enzyme gene expression compared with non-colitic group, probably associated with greater local vascularization and tissue basal membrane degradation (LI; VLODAVSKY, 2009). The disorder caused by TNBS instillation can be seen in scanning and transmission electron microscopy. In TNBS-control group, both in preventive and in curative groups, there was a disruption of the membrane and disarrangement of goblet cells and colonocytes, probably by heparanase actions. In the preventive protocol, the higher doses of *B. pilosa* (50mg/kg and 100mg/kg) were able to prevent heparanase gene expression enhance, leading to low membrane disruption. However, in curative protocol, only the intermediate dose, 50mg/kg hold this enzyme expression. According to HERMANO; LERNER; ELKIN (2012), heparanase represents a highly relevant, but equally challenging therapeutic target in inflammatory bowel disease and the effects of *B. pilosa* extract on the heparanase expression could represent a complementary product to the current drugs used to treat IBD.

The MAPKs are an evolutionary conserved family of serine/threonine kinases with a great number of substrates, which are involved in a wide range of biological

processes as growth, proliferation, differentiation, migration and apoptosis (CHANG, L.; KARIN, 2001; DHILLON et al., 2007). MAPK1 (ERK2) and MAPK3 (ERK1) are mitogen-activated protein kinases members of extracellular signal-regulated kinase family (ERK), which share nearly 85% amino acid identity in the core regions (LLOYD, 2006). Several authors described that these MAPK isoforms are functionally interchangeable, and a sufficient threshold of total MAPK activity is important for normal tissue development and function (LEFLOCH, R.; POUYSSEGUR; LENORMAND, 2008; LEFLOCH, RENAUD; POUYSSÉGUR; LENORMAND, 2009; UPADHYA; OGATA; RENEKER, 2013). When analyzed together, MAPK1/3 gene expression, in the preventive and curative protocol, were enhanced in colitic animals. Treatment with all doses of *B. pilosa* extract was able to decrease these gene expression levels. It has been demonstrated that MAPK1/3 was found in cells of the immune system and colonocytes during development and progression of human IBD (BROOM et al., 2009) and that a reduction in MAPK1/3 is significant because protects against inflammation and chronic pain (YU, 2012).

Colonic mucus layer has been reported to play a key role among the different factors that contribute to the maintenance of epithelial integrity (ALGIERI et al., 2013). In the mouse four Muc-type genes have been described in intestinal cells MUC1, MUC2, MUC3 and MUC4, which are divided in membrane-bound mucins (MUC1, MUC3, and MUC4) and gel-forming mucin (MUC2). MUC2 is the most prominent secretory mucin in the intestine with key role for maintenance of the mucus layer (EINERHAND et al., 2002; KIM, Y. S.; HO, 2010; TYTGAT et al., 1995).

In the preventive protocol, there was a MUC2 and MUC1 gene expression enhancement in TNBS-control group when compared with non-colitic animals. These results are in accordance with BOLTIN et al. (2013) that found an increase in goblet cell

differentiation and an upregulation of mucin synthesis in an acute intestinal inflammation. In contrary, there is no alteration in MUC3 and MUC4 gene expression after TNBS instillation. SHENG et al. (2012), in an elegant study, found no alteration in MUC3 and MUC4 mRNA in patients with UC. In our study, *B. pilosa* treatment in all doses diminished MUC1 and MUC2 gene expression caused by TNBS. Curiously, there is also an enhancement in MUC3 and MUC4 mRNA caused by *B.pilosa* extract in the dose of 25mg/kg. Upregulation of mucin allows that mucous protective layer to remain intact (BOLTIN et al., 2013).

On the other hand, in curative protocol, we observed no alterations in MUC1, MUC3 and MUC4 mRNA, but interestingly we detected a decrease in MUC2 expression in colitic animals when compared with healthy ones. This decrease is expected since chronic intestinal inflammation and infection leads to the ultimate depletion of goblet cells and reduction in mucin synthesis and secretion (BOLTIN et al., 2013). The administration of *B. pilosa* extract at the dose of 25mg/kg was capable to enhance the MUC1 mRNA, indicating the protective barrier function in the colonic mucosa. These effects may contribute to the restoration of the epithelial barrier, thus promoting TNBS colonic damage recovery (ALGIERI et al., 2013). According to NEURATH (2014b) it is potentially interesting for therapy of IBD to overcome the impaired barrier function by modulation of mucin function or expression.

There were some differences between gene expression and mucin production and release, mostly in preventive protocol presented in PAS/Alcian Blue staining. There are a plethora of mechanisms operating post-transcriptionally (even excluding mRNA processing events that take place in the nucleus) to regulate gene expression either specifically or globally. Expression of a gene can be controlled at many levels, including transcription, mRNA splicing, mRNA stability, translation and post-

translational events such as protein stability and modification (DAY; TUITE, 1998). So the difference found here could be explained based in one of these innumerous events that could have occurred after mRNA production.

In conclusion, *B. pilosa* extract improved ultra-structurally the colon tissue as demonstrated by electron microscopy besides diminishes inflammatory related genes. Indeed, we observed an improvement in protection mechanisms as evidenced by increased mucin expression. Altogether indicates that *Bidens pilosa* L. extract can be used to ameliorate the inflammation caused by TNBS and can be a potential source of compounds useful to treat IBD in humans.

Conflict of interest statement

The authors declare that there is no conflict of interest

Acknowledgement

Research in the Di Stasi lab (PhytoPharmaTech) is supported by São Paulo Research Foundation (FAPESP), grant number 11/50512-2. Fellowships: AEVQ from National Council for Scientific and Technological Development (CNPq - Brazilian Ministry of Science and Technology), LDAJ from Coordination for the Improvement of Higher Education Personnel (CAPES-Brazilian Ministry of Education), CARAC from São Paulo Research Foundation (FAPESP) and LCDS from National Council for Scientific and Technological Development (CNPq - Brazilian Ministry of Science and Technology). The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of financial support agencies.

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Figures Legends:

Figure 1: Electromicrographs and photomicrographs of colon samples from preventive protocol. Non-colitic group (1); TNBS-control group (2); 25mg/kg treated group (3); 50mg/kg treated group (4) and 100mg/kg treated group (5). In Scanning Electron Microscopy: crypts (white arrows), goblet cells (black arrows) and mucin (m). In Transmission Electron Microscopy: microvilli (black arrows), nucleus (n), goblet cells (G), mucin (m) and oedema (white arrows). In PAS/Alcian Blue Staining: Blue staining (white arrows), mucosa (m), submucosa (sm) membrane-bond mucins (black arrow) and oedema (oe).

Figure 2: Electromicrographs and photomicrographs of colon samples from curative protocol. Non-colitic group (1); TNBS-control group (2); 25mg/kg treated group (3); 50mg/kg treated group (4) and 100mg/kg treated group (5). In Scanning Electron Microscopy: crypts (white arrows), goblet cells (black arrows) and mucin (m). In Transmission Electron Microscopy: microvilli (black arrows), nucleus (n), goblet cells (G), mucin (m) and oedema (white arrows). In PAS/Alcian Blue Staining: Blue staining (white arrows), mucosa (m), submucosa (sm) membrane-bond mucins (black arrow) and oedema (oe).

Figura 1

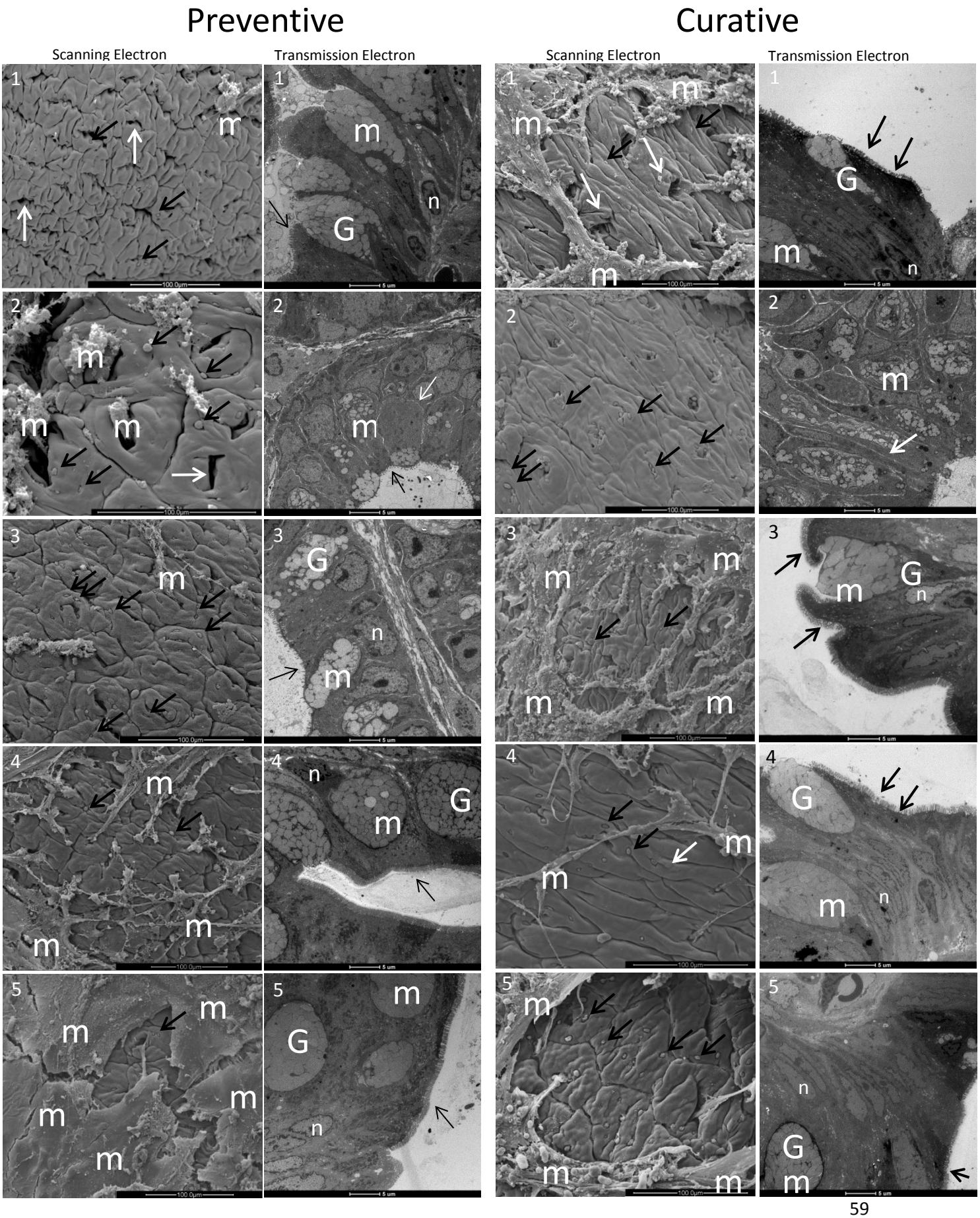
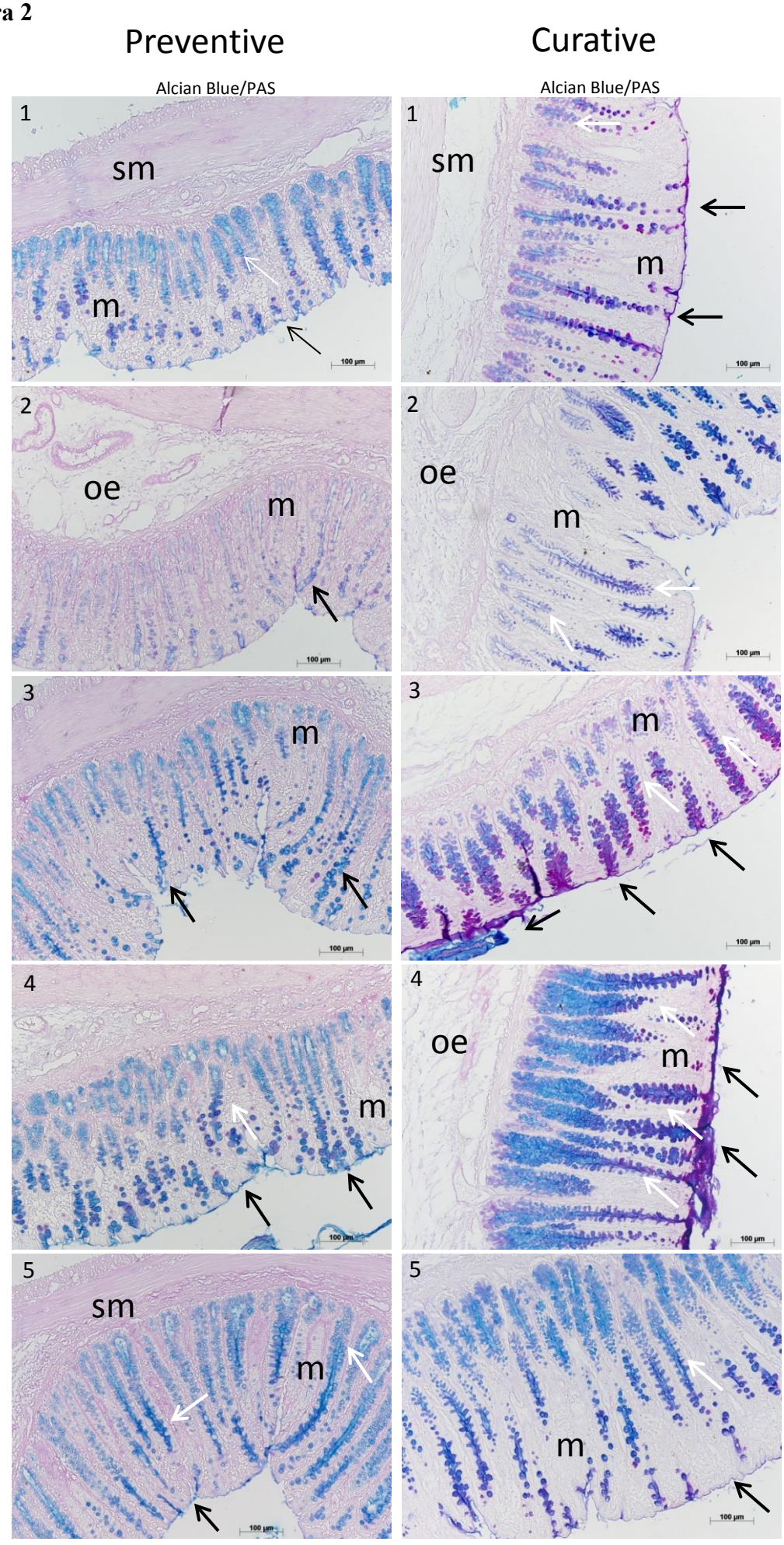

Preventive
Curative
Scanning Electron
Transmission Electron
Scanning Electron
Transmission Electron
1
2
3
4
5
m
G
n
100.0µm
5 µm

Figura 2


Preventive
Curative
Alcian Blue/PAS
Alcian Blue/PAS
1
sm
m
1
sm
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2
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3
m
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oe
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sm
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m

Table 1. Details of primers used for real-time PCR analysis

Target	Sequence	Annealing Temperature (°C)	Oligo Concentration (nM)	Fragment Size (bp)	NCBI Reference Sequence	cDNA concentration (µl)
GAPDH	**F** TGACTCTACCCACGGCAAGTTCAA **R** ACGACATACTCAGCACCAGCATCA	60	200	141	NM_017008.3	1
β-actina	**F** TTGCTGACAGGATGCAGAAGGAGA **R** ACTCCTGCTTGCTGATCCACATCT	60	100	159	NM_031144.2	1
HPRT	**F** AGGGAAGTGACAATCTACCTGACG **R** GAAATGTCTGTTGCTGCGTCCCTT	60	100	81	AA900579.1	0.125
HSP70	**F** ACTCCTTCGTTCGGTCTGCAATCA **R** CTGGGAATGCAAAGCACACGTGAA	60	200	92	NM_031971.2	0.125
Heparanase	**F** TGTCAAGAGTGAAAGGCCCAGACA **R** GCAGCTTCAAGTGCTTGGTGACAT	60	200	141	NM_022605.1	0.125
NF-κB	**F** AAACCAAAGCCCTGAAAGGCCATC **R** TCGGAAGGCCTCGAATGACATCAA	60	200	120	XM_342346.4	0.125
MAPK1	**F** AACAGGTTGTTCCCAAACGCTGAC **R** AGTCGTCCAGCTCCATGTCAAACT	60	200	187	NM_053842.1	0.5
MAPK3	**F** TACCTGGACCAGCTCAACCACATT **R** AGCAGGTCAAGAGCTTTGGAGTCA	60	200	173	NM_017347.2	0.5
MAPK6	**F** AACTGAGCCAGTGGAAGAAGGGAA **R** TTAACGTGGCCTGGATGGACTTGA	60	200	164	NM_031622.2	1
MAPK9	**F** TCATGGGAGAGCTGGTGAAAGGTT **R** ATGAACTCTGCGGATGGTGTTCCT	60	200	106	NM_017322.2 NM_001270544.1 NM_001270545.1	1
MUC1	**F** CCGCTACTACCAAGAACTGAAG **R** GAGCCTGACCTGAACTTGATAG	60	200	102	NM_012602.1	0.5
MUC2	**F** GGCTCTGCTCTCTGTGTTATAG **R** CAGTTTGGGAAGAAGGTAGGG	60	200	123	U68172.1	1
MUC3	**F** GGGAAATAGACCCTGCAGTTAG **R** GATCATCGCTTGCCGTCATA	60	200	107	U76551.1	0.125
MUC4	**F** ATGTGGAGGTGGGAGAAATG **R** CCCTGGAACTGGAATTAGAGAC	60	200	122	AF240632.1	0.5

Table 2. Effects of *Bidens pilosa* L. treatment on HSP70, Heparanase, MAPK3, MUC1, MUC2, MUC3 and MUC4 gene expression in colonic samples from preventive experiment.

	HSP70	Heparanase	MAPK3	MUC1	MUC2	MUC3	MUC4
Non-colitic	0.09 ± 0.01 **	0.17 ± 0.001**	0.43 ± 0.04***	0.18 ± 0.04**	8.11 ± 3.97*	0.14 ± 0.03	0,42 ± 0,14
TNBS-control	0.32 ± 0.07	0.44 ± 0.070	0.90 ± 0.09	0.39 ± 0.06	16.8 ± 1.23	0.13 ± 0.02	0,40 ± 0,09
Bidens 25	0.13 ± 0.01*	0.33 ± 0.08	0.01 ± 0.003****	0.09 ± 0.03***	7.31 ± 0.74*	0.86 ± 0.23 **	2,52 ± 0,70**
Bidens 50	0.30 ± 0.06	0.24 ± 0.02*	0.18 ± 0.09****	0.12 ± 0.03***	1.71 ± 0.54***	0.38 ± 0.11	0,76 ± 0,11
Bidens 100	0.07 ± 0.02**	0.15 ± 0.02**	0.02 ± 0.01****	0.002±0.001****	1.45 ± 0.23***	0.21 ± 0.04	1,50 ± 0,53

Data are expressed as the mean ± S.E.M. *p≤0.05; ** p≤0.01; *** p≤0.001; ****p≤0.0001

Table 3. Effects of *Bidens pilosa* L. treatments on HSP70, Heparanase, MAPK1, MUC1 and MUC2 gene expression in colonic samples from curative experiment.

	HSP70	Heparanase	MAPK1	MUC1	MUC2
Non-colitic	0.51 ± 0.04 **	0.62 ± 0.11*	0.84± 0.07*	0.67 ± 0.08	3.47 ± 1.27**
TNBS-control	1.12 ± 0.19	1.11 ± 0.22	0.99 ± 0.07	0.42 ± 0.03	0.32 ± 0.09
Bidens 25	0.64 ± 0.07**	0.75 ± 0.12	0.81 ± 0.01*	0.85 ± 0.11*	0.55 ± 0.13
Bidens 50	0.47 ± 0.04 ***	0.59 ± 0.10*	0.79 ± 0.04 **	0.49 ± 0.06	0.88 ± 0,173
Bidens 100	0.51 ± 0.08 **	0.82 ± 0.07	0.82 ± 0.03*	0.50 ± 0.08	0.49 ± 0,17

Data are expressed as the mean ± S.E.M. *p≤0.05; ** p≤0.01; *** p≤0.001

Conclusão

Com base no exposto:

- ✓ O extrato de *Bidens pilosa* L. possui atividade anti-inflamatória, evidenciada pela diminuição dos níveis de mieloperoxidase, IL-1β, IL-6, e TNF-α, além de modular os níveis de IL-10, citocina anti-inflamatória. Associado a esse efeito, apresentou também, atividade antioxidante pronunciada como demonstrado na prevenção da depleção dos níveis de glutationa, antioxidante endógeno.

- ✓ Além de alterar os níveis de proteínas importantes na inflamação, o extrato de *B. pilosa* foi capaz de modular a expressão de genes relacionados à inflamação, diminuindo a expressão de HSP70, heparanase e MAPK1/3 além de modular os níveis de mucinas, substâncias relacionadas à proteção do epitélio colônico.

- ✓ Sendo assim, o extrato supercrítico de *Bidens pilosa* L. representa uma fonte importante de compostos úteis no tratamento e prevenção da inflamação intestinal podendo complementar as terapias existentes atualmente na clínica.

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Anexo

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CERTIFICADO

Certificamos que o Protocolo nº 042/04-CEEA, sobre *"Inflamação intestinal aguda, crônica com recidiva induzida por Ácido trinitrobenzenosulfônico em ratos"* , sob a responsabilidade de **LUIZ CLAUDIO DI STASI**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado nesta data "Ad referendum" ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA).

Botucatu, 15 de junho de 2004.

Prof.Dr. Sílvio Luís de Oliveira
Presidente - CEEA

Nádia Jovêncio Cotrim
Secretária – CEEA