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ATIVIDADES ANTIULCEROGÊNICA GÁSTRICA E ANTIINFLAMATÓRIA INTESTINAL DE

Abarema cochliacarpos

Tese de Doutorado apresentada à Pós-Graduação do Instituto de Biologia para a obtenção do título de Doutor em Biologia Funcional e Molecular, área de Fisiologia.

Orientador(a): Prof(a). Dr(a). **Alba Regina Monteiro Souza Brito**

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Certificamos que o Protocolo nº **1287-1**, sobre **"Avaliação da atividade antiulcerogênica dos extratos de *Abarema cochiliocarpos* (Gomes) Barneby e Grimes e *Luffa cylindrica* L. Roem"**, sob a responsabilidade de **Profa. Dra. Alba Regina Monteiro Souza Brito / Maria Silene da Silva**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em reunião de **25 de junho de 2007**.

CERTIFICATE

We certify that the protocol nº **1287-1**, entitled **"Evaluation of the anti-ulcerogenic activity of *Abarema cochiliocarpos* (Gomes) Barneby e Grimes and *Luffa cylindrica* L. Roem extracts"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **June 25, 2007**.

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EPÍGRAFE

“Conquistar a si mesmo é uma tarefa maior que conquistar os outros”

(Buda Gautama)

SUMÁRIO

LISTA DE ABREVIATURAS

AC = fração butanólica do extrato metanólico de *A. cochliacarpos*

AF = fração acetato do extrato metanólico de *A. cochliacarpos*

AQF = fração aquosa do extrato metanólico de *A. cochliacarpos*

ALFAC = álcool-formaldeído-ácido acético

ANOVA = análise de variância uma via

bFGF = fator de crescimento fibroblástico básico

EGF = fator de crescimento epidermal

CCK_B = receptor de gastrina do tipo B

COX-1 = ciclooxigenase-1

COX-2 = ciclooxigenase-2

DAB = 3'3-diaminobenzidina

DAINEs = drogas antinflamatórias não esteroidais

DAG = diacilglicerol

DC = Doença de Chron

DII = Doenças Inflamatórias intestinais

ECL = células enterocromafins "like"

ESI/MS = espectrometria de massas com ionização por eletrospray

H^+,K^+-ATPase = bomba protônica de hidrogênio e potássio

H_2 = receptor histamínico do tipo 2

HE = hematoxilina-eosina

HGF = fator de crescimento de hepatócito

HSP-70 = chaperona-70

IL-1β = interleucina-1β

IL-10 = interleucina-10

iNOS = óxido nítrico sintase induzível

IP_3 = inositol 1,4,5-trifosfato

JNK = c-Jun N-terminal kinase

M_3 = receptor muscarínico do tipo 3

MAPK= proteína kinase ativada por mitógeno

MHC-II = complexo de histocompatibilidade principal

MPO = mieloperoxidase

NO = óxido nítrico

NOS = óxido nítrico sintase

i-NOS = óxido nítrixo sintase induzida

NP/PEG = produtos naturais / polietilenoglicol

NF-κB = fator nuclear kappa B

NOD2 = *Nucleotide-binding oligomerization domain*

PAF = fator ativador de plaquetas

PAS = ácido periódico de Shiff

PCNA = antígeno natural de proliferação celular

PDGF = Fator de Crescimento Derivado das Plaquetas

PGE_2 = prostaglandina E_2

PGH_2 = prostaglandina H_2

PGD_2 = prostaglandina D_2

PGI_2 = prostaglandina I_2

PGHS = prostaglandina H sintase

$PGF_{2\alpha}$ = prostaglandina $F_{2\alpha}$

PGs = prostaglandinas

RMN = Ressonância magnética nuclear

RU = Retocolite ulcerativa

SDS-PAGE = gel de poliacrilamida dodecil sulfato de sódio para eletroforese

TGI = Trato Gastrintestinal

Th = linfócito T *helper*

TNBS = ácido trinitrobenzenosulfônico

TNF-α = fator de necrose tumoral-α

VEGF = Fator de crescimento epitelial vascular

TXA_2 = tromboxano A_2

VIP = Peptídeo Intestinal Vasoativo

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RESUMO

Abarema cochliacarpos (Gomes) Barneby & Grimes, pertencente à família Mimosaceae, é conhecida popularmente como barbatimão por comunidades tradicionais do Nordeste do Brasil, que utilizam sua casca no tratamento da úlcera gástrica e inflamação, entre outros usos. Neste trabalho, inicialmente realizou-se a triagem da atividade antiulcerogênica de extratos e frações, utilizando-se o modelo de úlcera gástrica induzida por etanol absoluto em ratos. A fração butanólica do extrato metanólico, na dose de 150mg/kg, foi selecionada por ser a mais efetiva, a qual foi utilizada para o estudo dos mecanismos de ação antiulcerogênico em modelos de muco aderido e ligadura do piloro. Essa fração foi testada em modelo de atividade antiinflamatória intestinal em ratos. A toxicidade aguda de AC foi avaliada em camundongos Swiss (na dose de 5000 mg/kg) não tendo sido observados sintomas ou sinais de toxicidade. AC 150mg/kg foi sempre administrada oralmente aos animais, exceto no modelo de ligadura do piloro, no qual a via intraduodenal foi empregada. A fração foi capaz de aumentar significativamente o muco aderido, e alterar parâmetros bioquímicos da secreção ácida gástrica, produzindo redução do volume gástrico e aumento do pH, o que sugeriu possível atividade anti-secretória e citoprotetora. AC 150mg/kg, também apresentou atividade cicatrizante sobre lesões gástricas produzidas no modelo crônico de úlcera gástrica induzida por ácido acético a 30%, o que foi evidenciado pela redução da área de lesão, e confirmado na análise histológica das lesões. Indução da proliferação celular foi indicada pelos resultados de *proliferating cell nuclear antigen* (PCNA). A imunolocalização de *heat shock protein* (HSP-70), cicloxigenase-2 (COX- 2) e *vascular endothelial growth factor* (VEGF) no tecido gástrico sugeriu que a atividade de cicatrização da úlcera foi induzida por aumentos da expressão destes fatores, e da produção de muco, esta última evidenciada pelos resultados do PAS. No modelo de colite ulcerativa aguda induzida por ácido trinitrobenzenosulfônico-TNBS (10mg/animal), AC 100 e 150 mg/kg foram capazes de diminuir significativamente a área de lesão e os níveis de mieloperoxidase (MPO) na mucosa cólica. Porém, apenas AC 150 mg/kg diminuiu os níveis do fator de necrose tumoral alfa (TNF-α). A análise por western blot mostrou que AC 150 mg/kg induziu *down-regulation* da expressão de COX-2 e da enzima óxido nítrico sintase induzida (i-NOS), bem como demonstrou a redução da ativação de c-Jun N-terminal kinase (JNK). No modelo de colite crônica (TNBS, 30mg/animal) AC150 mg/kg demonstrou um perfil semelhante, ou seja, inibição dos parâmetros

inflamatórios do processo; porém, apenas neste modelo um aumento da produção da citocina antiinflamatória IL-10 foi evidenciada. Estes dados sugeriram um efeito protetor significativo da fração butanólica na fase crônica da resposta inflamatória associada à colite induzida por TNBS. O estudo fitoquímico de AC por espectrometria de massas (ESI/MS) e ressonância magnética nuclear (RMN) identificou catequinas como componente majoritário, sendo os taninos da classe das proantocianidinas. Tomados em conjunto, estes dados permitem concluir que AC150 de *Abarema cochliacarpos* possui compostos químicos com atividades antiulcerogênica gástrica e antiinflamatória intestinal, as quais podem ser atribuídas aos taninos e (+)-catequinas presentes nesta fração.

ABSTRACT

Abarema cochliacarpos (Gomes) Barneby & Grimes, which belongs the Mimosaceae family, is popularly known as "barbatimão" by the tradicional communities in Northeastern Brazil that use its bark in the treatment of gastric ulcer and inflammation, among other uses. In this work, the antiulcerogenic activity of its extracts and fractions was screened, using the gastric ulcer model induced by absolute ethanol in rats and selecting of the butanolic fraction of the methanolic extracts at dosis of 150 mg/kg as being in the most effective one. The action mechanisms of this fraction were studied based on the mucus gastric content and gastric secretion models. We have also decided to test it in anti-inflammatory intestinal activity model. Acute toxicity of AC150 was evaluated in Swiss mice, and no symptoms or visible toxicity signs were observed. Wistar rats were used in the others experiments. The intraduodenal pathway was utilized only in the pylorus ligature model; in the other models the oral pathway was employed. The AC150 was able to significantly increased the mucus production, as well as to change the biochemical parameters of the acid gastric secretion, reducing the gastric volume and increasing the pH, suggesting a cytoprotection and anti-secretory activity. AC150 showed healing activity in the chronic model of gastric ulcer induced by 30 % acetic acid. This was demonstrated by the reduction of lesion area, in agreement with HE histological analysis. The induction of cellular proliferation was indicated by the PCNA results. The immunolocation of HSP-70, COX-2 and VEGF in the gastric tissue suggested that the ulcer cicatrization activity was induced by the increased of the expression of these factors, as well as the increase of the mucus production highlightened in the PAS results. In the acute ulcerative colitis model induced by intra-colonic administration of trinitrobenzensulfonic acid (TNBS) (10 mg/animal) AC 100 and 150 mg/kg were able to significantly decrease the lesion and the mieloperoxidase (MPO) levels in the colonic mucosa. However, only AC150 decreased the levels of the tumour necrosis factor-alpha (TNF-α). The western blot analysis showed that AC 150 mg/kg induced the down-regulation of both cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) expression, and the involvement of signaling also demonstrated a reduction in the JNK activation in this dosis. In the chronic colitis model (TNBS, 30 mg/animal) AC 150 showed the same inhibition profile of the inflammatory parameters; nevertheless, the increase of the anti-inflammatory cytokine production IL-10 was highlightened only in this model. These data suggested

a significant protective effect of the chronic administration of AC during the chronic phase of the inflammatory response associated with TNBS induced colitis. Phytochemical studies by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) revealed that catechins were a major component into condensate class of tannins. Taken as a whole, these data allow us to conclude that the AC150 of *Abarema cochliacarpos* has chemical compounds that present antiulcerogenic gastric and anti-inflammatory activities, which may be attributed to the tannins and (+)-catechins of this fraction.

I Introdução

A procura por compostos bioativos de plantas medicinais permanece, na atualidade, como uma importante fonte para a descoberta de novos medicamentos justificando, assim, os numerosos estudos multidisciplinares existentes neste ramo da terapêutica; os esforços estão concentrados em sistematizar, padronizar e processar conhecimentos empíricos, baseados em compostos vegetais locais, que requerem estudos mais aprofundados antes de serem incorporados à prática médica assistencial.

O metabolismo secundário dos vegetais origina compostos importantes para sua interação com micro e macrorganismos, bem como para proteção contra fatores ambientais estressantes. Quando ingeridos, determinados metabólitos de plantas comestíveis interagem beneficamente com domínios regulatórios de proteínas funcionais compartilhados por plantas e animais, que foram conservados pela evolução das espécies (Shapiro et al., 2007).

Informações do conhecimento tradicional de grupos étnicos sobre plantas medicinais e seus usos desempenham papel vital na descoberta de novos produtos de plantas como agentes terapêuticos (Almeida, Navarro & Barbosa-Filho, 2001). Pesquisas pré-clínicas confirmam a eficácia de várias plantas medicinais no tratamento de doenças gastrintestinais, sendo que suas propriedades terapêuticas são atribuídas a diferentes compostos orgânicos e inorgânicos como alcalóides, cumarinas, terpenóides, taninos, ácidos fenólicos e micronutrientes antioxidantes, como cobre e manganês (Chechinel-Filho & Yunes, 2001; Mota et al, 2009).

Espécies vegetais da Caatinga são ricas em compostos fenólicos, que aparecem em elevadas concentrações em algumas das espécies intensivamente usadas por comunidades locais; e essas substâncias correlacionam-se com indicações terapêuticas recorrentes (Monteiro et al., 2006), como é o caso das espécies conhecidas popularmente como "barbatimão", destacando-se aquelas do gênero *Stryphnodendron* (*Stryphnodendron adstringens* (Martius) Coville, *Stryphnodendron polyphyllum* e *Stryphnodendron obovatum*). Estudos científicos já têm confirmado algumas destas atividades (Audi et al., 1999; Martins et al., 2002, Souza et al., 2007). Outras espécies, entretanto, como *Dimorphandra mollis* Benth (Santos et al., 2002) e *Abarema cochliacarpos* (Gomes) Barneby & Grimes (Silva et al., 2006; Silva et al., 2009) também são conhecidas como "barbatimão" e usadas para os mesmos propósitos terapêuticos, sem os respectivos estudos comprobatórios da alegada atividade farmacológica. Dessa forma é fundamental investigar cientificamente as espécies de "barbatimão", para que sejam definidos os perfis químico e farmacológico de cada uma delas.

Assim, explorar novos alvos farmacológicos de proteção do trato gastrintestinal, provenientes de plantas medicinais, é importante, de vez que, mesmo existindo no mercado drogas que levam à remissão completa das úlceras gastroduodenais, a recidiva destas lesões mantém-se como importante questão a ser investigada. Em relação às Doenças Inflamatórias Intestinais (DII) não é diferente; enquanto não se consegue identificar suas causas, o tratamento dessa doença baseia-se no controle dos fenômenos inflamatórios que ocorrem no processo, o que não propicia remissão da doença (Royero, 2003).

1 - Úlcera péptica

Úlceras são definidas como rompimento da mucosa do trato gastrintestinal (TGI), provocando lesão que penetra através da muscular da mucosa, chegando à submucosa ou atingindo tecidos mais profundos. Embora possa ocorrer em qualquer ponto do TGI, prevalecem no estômago e duodeno (Cotran, Kumar & Robbins, 1994). O estresse psicológico, o hábito de fumar, o consumo de álcool, o uso de drogas antiinflamatórias não esteroidais (DAINEs), incluindo aspirina, imunossupressores e o declínio, relacionado à idade, dos níveis de substâncias protetoras da mucosa como muco, bicarbonato e as prostaglandinas (PG), são fatores que contribuem para a doença ulcerosa péptica. Entretanto, foi reconhecido que a infecção por *Helicobacter pylori* é uma das causas mais importantes de ulceração duodenal e gástrica (Yuan, Padol & Hunt, 2006).

A razão do surgimento das úlceras pépticas não é bem compreeendida. Acredita-se existir um desbalanço entre os fatores agressores (HCl e pepsina) e protetores (PG, muco e bicarbonato) da mucosa (Brzozowski, 2003).

O ácido clorídrico é produzido nas glândulas gástricas do corpo e fundo do estômago numa concentração de 150 a 160 mEq/L. A função primária destas células é produzir HCl, processo que possui como enzimas centrais a anidrase carbônica e a H^+,K^+-adenosina trifosfato (H^+,K^+-ATPase), cuja atividade é regulada por vários fatores neuroendócrinos que regulam a secreção ácida. Também produz fator intrínseco, importante na absorção da vitamina B_{12} (Collares-Buzato & Arana, 2005).

A secreção ácida gástrica é regulada por diversos mediadores, que agem diretamente ou indiretamente nas células parietais (Schubert, 2005). Os principais secretagogos de HCl são acetilcolina, neurotransmissor parassimpático vagal, gastrina, liberada das células G antrais, e histamina, liberada das células enterocromafins (ECL), que atuam respectivamente sobre os

receptores muscarínicos (M_3), CCK_B e H_2, localizados na membrana basolateral da célula parietal. Todos estes receptores estão acoplados a diferentes proteínas G (Curi et al., 2009).

Os receptores para acetilcolina e gastrina estão acoplados à proteína Gαq, que liberando cálcio dos reservatórios intracelulares para o citosol, ativa proteínas quinase dependentes de calmodulina (PKC); também aumentam os níveis de diacilglicerol (DAG) que também ativam PKC. A histamina, por sua vez, ativa a proteína Gαs, acoplada à adenilato ciclase da membrana, aumentando os níveis de AMPcíclico. Ambos os segundos mensageiros, Ca^+ intracelular e AMPc, ativam diversas quinases e esse processo culmina com a translocação da H^+,K^+-ATPase das membranas tubulovesiculares da célula parietal, e dos canais para cloro, para a superfície celular apical, resultando em secreção ácida para o lúmen (Schubert, 2005; Curi et al., 2009; Collares-Buzato & Arana, 2005).

O principal inibidor da secreção ácida gástrica é a somatostatina, que no estômago é liberada das células D do corpo e do antro gástrico (Dimaline & Varro, 2007). Atua inibindo a secreção de ácido agindo diretamente nas células parietais e indiretamente por inibir a secreção de histamina e de gastrina (Hou & Schubert, 2006). Suas ações são mediadas por receptores acoplados à proteína G_i, sendo o subtipo SSTR2 o regulador predominante da secreção ácida gástrica (Schubert, 2005). O receptor de somatostina, acoplado à proteína G_i, inibe a via acoplada à Gαs, e, em algumas células, como às ECL, inibem também a via acoplada à Gαq. A inibição desta última via explica o efeito do receptor SST2 no bloqueio da estimulação da secreção ácida pela gastrina (Athman et al., 2000).

O estudo dos mecanismos relacionados à defesa da mucosa gástrica ganhou maior impulso quando da descoberta de que apenas uma minoria (15%) dos pacientes infectados por *Helicobacter pylori* desenvolviam úlcera gástrica (Walsh & Peterson, 1995), deixando claro que fatores relacionados ao hospedeiro são de fundamental importância para que o indivíduo desenvolva ou não esta doença.

Estes mecanismos envolvem fatores funcionais, humorais e neurais. A secreção de muco, a microcirculação e a motilidade gastrintestinal são fatores funcionais, enquanto que prostaglandinas, e óxido nítrico agem como fatores humorais; neurônios sensoriais sensitivos agem como fatores neurais. Proteção à curto prazo, conhecida como citoproteção adaptativa, foi originalmente introduzida por Robert e colaboradores (1978) para descrever a ação protetora a certos agentes

irritantes, como etanol 20% e NaCl 5%; esta ação protetora é atribuída à ação de prostaglandinas e óxido nítrico.

Dentre os fatores funcionais de defesa da mucosa, destaca-se o papel da barreira muco-bicarbonato-fosfolipídeo. Esta barreira contínua é formada por muco e bicarbonato, secretado pelas células epiteliais superficiais e pelos fosfolipídios surfactantes que as recobrem. Assim, essas células são hidrofóbicas, repelindo ácido e agentes lesivos solúveis em água. O bicarbonato mantém um microambiente neutro (pH 7.0), impedindo a retrodifusão de ácido e pepsina e a digestão proteolítica da superfície do epitélio (Laine, Takeuchi, & Tarnawski, 2008).

O muco gelatinoso é secretado por expulsão apical, sendo uma secreção espessa formada por aproximadamente 95% de água e 5% da proteína mucina, produto do gene MUC, além de eletrólitos. Mucinas são os principais componentes da resposta imune inata da mucosa do trato gastrintestinal (Dey, Lejeune & Chadee, 2006). Quimicamente, o muco é bastante resistente ao ataque das enzimas gastrintestinais e, como resultado, essa estrutura mantém a lubrificação da mucosa, a diferença de pH entre a superfície da mucosa e o lúmen gástrico, além de favorecer fluxo unidirecional de íons hidrogênio, neutralizando o ácido do suco gástrico. Dessa forma, a parede gástrica subjacente *normal* nunca é diretamente exposta à secreção gástrica proteolítica e altamente ácida (Aires et al, 2008; Laine, Takeuchi, & Tarnawski, 2008).

Uma contínua camada de muco aderente no estômago, duodeno e partes distais do TGI tem sido demonstrada experimentalmente. Através de um método envolvendo microscopia confocal e microscopia intravital, observou-se uma camada de muco gel aderente na superfície da mucosa, no corpo, no antro e no duodeno de ratos anestesiados. Porém, o que mais chamou a atenção é que duas formas físicas do muco puderam ser observadas: uma, fracamente aderente à camada mucosa, poderia ser removida por sucção e teria função lubrificante; a outra, fortemente aderida, com função protetora, permanecia (Allen & Flemström, 2005). A estrutura e a espessura do gel são fatores determinantes na eficácia desta barreira protetora. As unidades formadoras da mucina polimerizam-se em mucinas multiméricas, essenciais para a formação do gel (Allen & Snary,1972), pois seu comprimento e estabilidade estão diretamente relacionados à porcentagem de mucinas multiméricas em relação às monoméricas (Sellers et al., 1988).

A estrutura desta camada pode ser comprometida por estados patológicos como infecção por *Helicobacter pylori*, úlcera gástrica e colite ulcerativa, durante as quais a estrutura e a espessura do muco são afetadas, ocorrendo diminuição da forma multimérica da mucina na camada de muco

aderente, tornando-a mais frágil e menos estável (Atuma et al., 2001). Substâncias ulcerogênicas como aspirina e sais biliares, causam dissipação da camada mucosa e fosfolipídica, levando a retrodifusão do ácido e a lesão da mucosa (Allen & Flemström, 2005).

A presença de bicarbonato numa camada de muco aderente estável cria um gradiente de pH na superfície do estômago e duodeno, provendo a primeira linha de defesa contra o ácido luminal. O bicarbonato é produzido pelas células superficiais do estômago e pelas glândulas de Brünner no duodeno; permanece em grande parte abaixo ou na camada mucosa (Aires et al., 2008). Estudos experimentais de gradiente de pH provêm evidências da existência da barreira muco-bicarbonato *in vivo* e a presença de um pH próximo à neutralidade na superfície epitelial (Laine, Takeuchi, & Tarnawski, 2008). A secreção de muco é estimulada por hormônios gastrintestinais, incluindo gastrina e secretina, como também PGE_2 e agentes anticolinérgicos (Allen & Flemström, 2005).

Vários estudos têm mostrado que o aumento do fluxo sangüíneo, ocorrido durante elevação dos níveis de secreção ácida, pode ser um mecanismo de defesa da mucosa a estímulos nocivos. A redução do fluxo sangüíneo da mucosa gástrica exacerba lesões por agentes ulcerogênicos, enquanto pouco ou nenhum dano ocorre depois da ação de agentes necrotizantes se o fluxo sangüíneo for mantido em níveis apropriados, juntamente com a citoproteção das prostaglandinas (Abdel-Salam et al, 2001).

Outro componente dos mecanismos de defesa da mucosa são as *heat shock proteins* (HSP), que são um grupo de proteínas altamente conservadas expressas pelas células epiteliais em resposta ao estresse (Tsukimi et al., 2001). Sua principal função é promover a tolerância contra fatores estressantes, mantendo a homeostasia celular, bem como aumentando sua resistência contra agentes lesivos (Chang et al., 2007). Estão envolvidas em eventos celulares, como empacotamento, montagem e transporte de proteínas, que protegem as células dos efeitos citotóxicos da agregação protéica produzida por vários agentes estressantes (Konturek et al., 2008). HSP70 é uma das mais importantes HSPs na proteção da mucosa gástrica. A superexpressão da HSP-70 tem sido observada em ratos com úlcera gástrica aguda e crônica, gastrite atrófica crônica e em pacientes com câncer gástrico. Esta superexpressão de HSP-70 promove a cura da úlcera por aumentar o fluxo sanguíneo da mucosa e a multiplicação celular (Tsukimi, Okabe, 2001).

O nível final de defesa da mucosa é ativado quando uma úlcera se forma. A úlcera resulta de necrose tecidual desencadeada por isquemia da mucosa, formação de radicais livres e inibição da

condução de nutrientes. Todo este processo é desencadeado por lesão microvascular e vascular, como trombose, constrição e outras oclusões (Tarnawski, Hollander & Stachura, 1990).

Histologicamente, uma úlcera consiste de duas características principais: margem da úlcera, formada pela mucosa adjacente não necrótica, que é o componente epitelial; tecido de granulação, que é componente do tecido conectivo. Este último consiste de fibroblastos e macrófagos da proliferação de células endoteliais que formam microvasos (Tarnawski, 2005). Nesse momento, a úlcera é reparada através de crescimento e formação de glândulas gástricas, crescimento de novos vasos sanguíneos (angiogênese), reinervação da mucosa por nervos intrínsecos e extrínsecos, além de deposição de matriz extracelular; todos os eventos conduzem à formação da cicatriz (Wallace & Ma, 2001; Tarnawski, 2005). São controlados por fatores de crescimento, fatores de transcrição e citocinas.

Os fatores de crescimento são os principais estimuladores da proliferação, divisão, migração e re-epitelização (Cotran, Kumar & Robbins, 1999). A ulceração desencadeia nas células da superfície da mucosa, da margem da úlcera, a produção de VEGF, *epidermal growth factor* (EGF), *basic fibroblast growth factor* (bFGF) e *hepatocyte growth factor* (HGF), trefoil peptides e COX-2, de forma sincronizada espacial e temporalmente (Milani & Calabro, 2001). Estes fatores produzidos localmente ativam a proliferação e estimulam a migração celular, por induzirem rearranjos no esqueleto de actina, por vias endócrinas e parácrinas (Tarnawski, 2005).

Dentro de 48 a 72 horas após a ulceração desenvolve-se na base da úlcera o tecido de granulação (Cotran, Kumar & Robbins, 1999). Este tecido é composto por macrófagos, fibroblastos e vasos neoformados que estão suportados por uma matriz frouxa de fibronectina, ácido hialurônico e colágenos tipos I e II (Guidugli-Neto, 1992); o tecido caracteriza-se pela presença de muitos espaços vazios devido à imaturidade dos vasos que, ao serem observados a olho nu, parece conter muitos grânulos na superfície. A neovascularização é essencial neste estágio porque permite a troca de gases e a nutrição das células metabolicamente ativas (Balbino, Pereira & Curi, 2005), permitindo formação da microvasculatura dentro da cicatriz da úlcera.

Angiogênese é a formação de uma nova rede vascular, sendo essencial para cura de úlceras crônicas gastroduodenais. Este processo ocorre por uma série de etapas, as quais incluem: a) degradação de capilares da membrana basal por metaloproteinases da matriz; b) migração e proliferação de células endoteliais no espaço perivascular; c) formação de tubos microvasculares

seguidos por anastomose; d) estabelecimento das membrana basal e luminal; e d) formação de nova rede microvascular (Cotran, Kumar & Robbins, 1999; Folkman & Amore, 1996; Tarnawski, 2005).

O crescimento do tecido de granulação e a formação de novos microvasos através da angiogênese estimulada por bFGF, VEGF, PDGF, angiopoietinas, e possivelmente outros fatores de crescimento e citoquinas IL-1 e TNF-α (Tarnawski, 2005) é essencial na resolução do processo. Dentre os peptídeos angiogênicos que têm sido identificados, VEGF é o mitógeno específico das células endoteliais, porque seus receptores VEGF-R1 ou VEGF-R2 são primariamente restritos às células endoteliais; sua ligação aos receptores inicia a fosforilação de numerosas proteínas citosólicas envolvidas na transdução do sinal que desencadeia proliferação de células endoteliais, migração e formação de tubos microvasculares (Ferrara, 2004).

A terapêutica da úlcera gástrica inclui basicamente, controle farmacológico da secreção ácida gástrica. Os histaminérgicos, como cimetidina e ranitidina, bloqueiam competitivamente as ações da histamina em todos os receptores H_2; porém sua principal ação clínica na inibição da secreção de ácido gástrico, ou seja, essas drogas inibem a secreção ácida estimulada por histamina e gastrina, além de reduzirem a secreção ácida estimulada por acetilcolina. As drogas anti-H_2 promovem cicatrização das úlceras duodenais, conforme evidenciado em estudos clínicos (Welage, 2005.

A identificação da enzima H^+,K^+-ATPase como via final de secreção ácida gástrica forneceu a oportunidade de desenvolver uma outra classe de agentes terapêuticos que inibem a secreção ácida gástrica, os inibidores da bomba de prótons. Quando comparados com outros inibidores da secreção ácida gástrica, estes têm se mostrado mais efetivos (Jonhson, 2003). O derivado benzimidazólico omeprazol e o mais recentemente desenvolvido lansoprazol, inibem a secreção ácida por agirem na H^+,K^+-ATPase. Estas pró-drogas são convertidas em formas ativas nos canalículos secretórios das células parietais e os inibidores ativados reagem com SH (grupo tiol) da bomba de prótons, resultando em inibição da formação de ácido (Chandranath, Bastaki & Singh, 2002). Esta ligação covalente inibe a ATPase, tornando a secreção ácida dependente da síntese *de novo* da bomba. Em ratos, a potência do lansoprazol têm se mostrado duas vezes maior que a do omeprazol (Schubert, 2005). Como o mecanismo de troca hidrogênio-potássio é o passo final na formação da secreção ácida, os inibidores da bomba de prótons são considerados mais potentes agentes anti-secretórios que a histamina, agonista do receptor H_2, tendo uma ação prolongada e uma maior capacidade de curar a úlcera (Garner et al.,1996).

A combinação da terapia anti-secretória com a antibioticoterapia tem sido a terapia de escolha para a erradicação do *Helicobacter pylori*. Existem vários mecanismos pelos quais os inibidores da bomba de prótons podem aumentar a eficácia dos antibióticos. A inibição da secreção ácida torna os antibióticos mais estáveis no ambiente menos ácido, aumentando assim a concentração de antibióticos no suco gástrico, como também o aumento da sensibilidade do *H. pylori* aos antibióticos (Hunt, 2005).

No entanto, a despeito de todos os avanços na terapêutica da úlcera gástrica, questões fundamentais permanecem sem resposta. Necessita-se: a) encontrar novas formas de erradicação do *H. pylori*, num momento de aumento das taxas de falha de erradicação; b) desenvolver melhores métodos que impeçam o desenvolvimento e recorrência da úlcera gástrica em usuários de DAINEs e c) descobrir qual o melhor tratamento para úlceras pépticas com causas não associadas à *H. pylori* nem às DAINEs.

2 Doenças Inflamatórias Intestinais

Esta condição inclui uma série de distúrbios multisistêmicos, de etiologia desconhecida, caracterizada por inflamações recorrentes do trato digestivo e cujos quadros clínicos mais representativos são a Doença de Chron (DC) e a Retocolite ulcerativa (RU) (Montero, 2007). Propõe-se que DII seja uma resposta imunitária descontrolada frente a um estímulo não identificado até o momento, que ocorre em um indivíduo geneticamente predisposto (Abraham & Cho, 2009).

Na DC ocorre uma inflamação transmural, assimétrica e, ocasionalmente, granulomatosa, que geralmente afeta o íleo e o cólon, mas podendo afetar qualquer outro segmento do aparelho digestivo, desde a boca até o ânus, bem como causar complicações extraintestinais e sistêmicas. Na RU esta inflamação é superficial, simétrica, contínua, quase exclusivamente localizada no reto e cólon, na qual também há potencialmente comprometimento sistêmico (Baumgart & Sandborn, 2007).

Há cada vez mais evidências de que a DC e RU representam um grupo heterogêneo de doenças que possuem como evolução final comum a inflamação da mucosa (Royero, 2003). Estudos com modelos animais sugerem claramente resposta imunitária descontrolada do hospedeiro, contra antígenos bacterianos da flora intestinal normal (Montero, 2007; Fantini et al, 2007), é facilitada

provavelmente por defeitos na função de barreira do epitélio intestinal e do sistema imune da mucosa (Podolsky, 2002). Apesar de DC e RU apresentarem diferentes processos patofisiológicos, compartilham vias não específicas que têm como estágio final a lesão tecidual (Fantini et al., 2007).

Embora a incidência e prevalência de doenças inflamatórias intestinais comecem a estabilizar em áreas de elevada incidência, como no Norte da Europa, Reino Unido e América do Norte, que são regiões tradicionalmente associadas às DII, são 2, 2 e 1,4 milhões de pessoas, na Europa e nos Estados Unidos, respectivamente, que sofrem destas doenças. Baixas taxas de incidência são relatadas para América do Sul, Sudeste da Ásia e África (com exceção da África do Sul), e Australia. Embora esses dados sugiram um gradiente Norte-Sul, eles podem também indicar variações no acesso e na qualidade dos serviços de saúde, como também diferenças existentes no nível de industrilização, saneamento e higiene (Loftus, 2004).

Várias observações clínicas sugerem que fatores genéticos contribuem para a susceptibilidade à DII. Os familiares em primeiro grau de indivíduos afetados têm risco de 25 a 50 e 10 a 20 vezes, de desenvolver DC e RU, respectivamente, quando comparados com à população em geral (Zheng et al, 2003). Estudos genéticos têm destacado a importância da interação micróbio-hospedeiro na patogênese das DII. As descobertas que mais se destacam nesta área estão centradas nas regiões genômicas que contem o *nucleotide oligomerization domain* 2 (NOD2), um gene autofágico. Autofagia é um mecanismo de eliminação de componentes intracelulares, incluindo organelas, corpos apoptóticos e microrganismos (Levine, 2007). A proteína NOD2 é um sensor intracelular de peptidoglicanos bacterianos e permite a autofagia das células, regulando e degradando diversos componentes intracelulares, incluindo patógenos. A associação entre DC e o gene NOD2 inclui três polimorfismos deste gene que alteram a sequência de aminoácidos NOD2, prejudicando a respostas ao peptidoglicano. Entretanto, apenas os polimorfismos de NOD2 não são suficientes para causar a Doença de Chron, o que é indicativo da complexidade desta desordem multifatorial (Abraham & Cho, 2009).

Pelo menos 7 *loci* têm sido identificados (*Inflammatory Bowel Disease* 1-7- IBD 1-7) nos cromossomos que se relacionam a genes de susceptibilidade, estando as mutações centradas nos genes NOD2/CARD15 (*nucleotide oligomerization domain 2/caspase recruitment domain 15*), no complexo de histocompatibilidade principal (MHC-II) e em genes de citocinas, de receptores de citocinas e de moléculas de adesão (Zheng et al, 2003, Poldoski, 2002).

Os fatores ambientais também desempenham um importante papel no desenvolvimento da DII, concordando com o aumento da incidência durante a segunda metade do século XX, como resultado de profundas mudanças no estilo de vida nos países desenvolvidos. Tabagismo, dieta, drogas, estresse e microrganismos são fatores ambientais reconhecidos como de risco (Montero, 2007).

O hábito de fumar cigarros está associado à menor exacerbação de RU; no entanto, para a DC, o cigarro agrava o curso da doença, promove a formação de fístulas e estenoses, aumenta as taxas de exacerbação e a necessidade de utilização de corticosteróides (Ingram et al., 2005). Estudos experimentais sugerem que os benefícios da nicotina na RU são devido ao aumento da produçao de muco, diminuição da produção de citocinas pró-inflamatórias e de óxido nítrico e a melhora da função de barreira intestinal. Já os efeitos prejudiciais da nicotina sobre a doença de Chron podem ser atribuídos ao aumento no influxo de neutrófilos na mucosa intestinal (Baumgart & Sandborn, 2007).

Quanto ao papel da nutrição ou a intervenção da dieta nas DII, uma revisão de estudos caso-controle do consumo diário de alimentos por pacientes com DII, realizada por Asakura et al. (2008) mostrou que o consumo de carne, açúcar e doces aumentou o *odds ratio* da ocorrência de DC e RU. Os alimentos significantemente associados à DC foram carne, peixe, lipídios, açúcar e doces; para a RU, carne, açúcar, doces e alimnetação de *fast food*. Fortalecendo a hipótese de nutrição enteral em pacientes com DII, polifenóis e outros elementos, incluindo probióticos, ácidos graxos de cadeia curta e fatores de crescimento podem desempenhar importante papel como uma fórmula específica na DC, cujos resultados mostraram que a dieta enteral é efetiva como indutora de remissão (Royero, 2003; Shapiro, 2007).

Diversos estudos têm evidenciado que a microbiota luminal é um requisito, e talvez o fator central, no desenvolvimento da DII. Esta inferência é suportada por estudos em modelos murino de colite estabelecidos por observações genéticas e reforçados por observações clínicas (Podolski, 2002). Por exemplo, o desenvolvimento de colite espontânea em ratos e camundongos parece requerer a presença da flora luminal; colite não se desenvolve em cepas mutantes quando elas são mantidas em ambiente *germ-free*, mas se desenvolve rapidamente quando estes camundongos são colonizados por bactérias comensais (Rath et al., 2001).

O efeito conjunto dos fatores genéticos e ambientais sobre a DII é a ativação sustentada da resposta imune da mucosa. Porém, não está claro se o sistema imune é ativado como resultado de

um defeito intrínseco, sendo uma ativação constitutiva ou falha de mecanismos de *down- regulation*, ou se o estímulo continuado é resultante de alterações na barreira epitelial da mucosa (Poldoski, 2002).

O epitélio intestinal é a interface entre a microbiota intestinal e o tecido linfóide associado com o sistema gastrintestinal, desempenhando um papel crítico na moldagem da barreira imune da mucosa à excessiva entrada de bactérias e outros antígenos do lúmen intestinal para dentro da circulação (Abraham & Cho, 2009). As junções intercelulares são as estruturas responsáveis pela união do espaço entre as células epiteliais adjacentes (espaço paracelular) e as *tight junctions* que são os elementos-chave da união entre as células. Na DII, o espaço paracelular tem a permeabilidade aumentada e a regulação das *tight junctions* é defeituosa. Estas anormalidades podem ser decorrentes de um defeito primário da função de barreira ou serem resultantes da inflamação (Turner, 2006).

A complexa população de células imune da lâmina própria tem como função equilibrar o requerimento da tolerância imune à microbiota intestinal com a necessidade de defesa contra patógeno. A característica fundamental da DII é a acentuada infiltração na lâmina própria de células do sistema imune inato (neutrófilos, macrófagos, células dendríticas e célula T *natural killer*), e do sistema imune adaptativo (células B e células T) (Abraham & Cho, 2009). O aumento da quantidade, bem como ativação destas células na mucosa intestinal, eleva a produção de uma grande variedade de mediadores não específicos da inflamação, incluindo TNF-α, interleucina-1β, interferon-γ, quimiocinas, fatores de crescimento, como também prostaglandinas, leucotrienos e espécies reativas do nitrogênio, como o NO (Podolski, 2002). Estes mediadores aumentam o processo inflamatório e a destruição tecidual, o que pode levar às manifestações clínicas da doença (Abraham & Cho, 2009; Podolski, 2002).

Células epiteliais, células de Paneth, macrófagos, células dendríticas e células endoteliais expressam NOD2 (Abraham & Cho, 2006), em resposta à estimulação produzida por peptidoglicanos bacterianos o que ativa o fator nuclear kappa B (NF-κB) e a via de sinalização *mitogen-activated protein* (MAP) *kinase*, que leva à produção de citocinas e peptídeos antimicrobianos (Abraham & Cho, 2006). O NF-κB é um fator de transcrição pleiotrópico, ativado por uma grande variedade de estímulos. Usualmente está ligado e inativado pelo inibidor citoplasmático do κ B (IkB), porém, estímulos como endotoxinas e citocinas inflamatórias (TNF-α, por exemplo), estresse oxidativo, bactérias e vírus podem deslocá-lo para o núcleo, iniciando o

processo transcricional (Alárcon De La Lastra, 2006; Neumann & Neumann, 2007). O NF-κB é o principal regulador da expressão induzida de genes envolvidos nas respostas imune e inflamatória intestinais, como TNF-α, i-NOS e COX-2, molécula de adesão intercelular e ativação de linfócitos, monócitos/macrófagos e células endoteliais induzida pela inflamação (Shapiro et al., 2007).

O acúmulo de leucócitos é outra característica da DII. Seu recrutamento e aderência aos microvasos ocorrem através da ação de TNF-α e interleucina-1β, que estimulam a *up-regulation* de moléculas de adesão (selectinas e integrinas) nas células endoteliais vasculares (Hatoum, Heidemann, Binion, 2006).

A resposta imune inata desempenha papel fundamental na DII. Este nível de defesa é formado por mastócitos e macrófagos residentes dentro da lâmina própria, que agem como sentinelas; quando detectam a entrada de substâncias estranhas na mucosa, como antígenos e endotoxinas, respondem através da liberação de mediadores químicos que coordenam uma resposta inflamatória apropriada (Wallace & Ma, 2001).

A secreção de citocinas por células apresentadoras de antígenos, como células dendríticas e macrófagos ativados, orquestram muitos dos processos inflamatórios envolvidos nesta enfermidade, além de desencadear a resposta imune adaptativa, através da ativação e diferenciação dos linfócitos T helper (Th) (Sanchez-Muñoz et al., 2008).

Citocinas, mediadores polipeptídicos produzidos principalmente por células do sistema imune, desempenham papel importante na regulação do sistema imune da mucosa; muito do que se sabe sobre elas provém de suas ações no intestino delgado e grosso, devido a sua importância na patogênese das DII. Estes mediadores se apresentam como reguladores–chave da resposta imune da mucosa intestinal, sendo conhecidos por participarem do rompimento do estado de equilíbrio de inflamação controlada ou inflamação fisiológica do intestino (Wallace & Ma, 2001; Jump & Levine, 2004).

A diferenciação de linfócitos Th CD4+ na subpopulação Th1, que produz grande quantidade de citocinas pró-inflamatórias como fator de necrose tumoral- α (TNF-α), interleucina-1β (IL-1β) e interleucina-12 (IL-12) têm papel fundamental no desenvolvimento da Doença de Chron, enquanto que na colite ulcerativa predomina a expressão da subpopulação Th2, envolvidas na resposta humoral associada às interleucinas IL-4, IL-5, IL-6, IL-10 e ao TNF-α (Podolsky, 2002). Estudos revelam que o desequilíbrio entre citocinas pró-inflamatórias, como IL-1, IL-1β, TNF-α, INF-γ, e anti-

inflamatórias, IL-4, IL-10, IL-11, IL-13, desempenham um papel essencial na patofisiologia da DII (Fantini, et al. 2007).

Dentre as citocinas pró-inflamatórias, TNF-α é a citocina-chave, que desencadeia cascata de reações pró-inflamatórias, estimulando a produção de numerosas citocinas, sendo importante tanto na indução como na sustentação da inflamação (Owczarek, et al. 2009). Pode ser produzido e liberado pela ativação de monócitos, macrófagos, células T e mastócitos; também regula a expressão de moléculas de adesão na superfície da membrana endotelial, como principal mediador da inflamação (van Deventer, 1997). TNF-α é importante em doenças autoimune como a DII e níveis aumentados podem ser encontrados no local da inflamação, tanto como no sangue, quanto nas fezes e tecido intestinal de pacientes com DII (Murch et al. 1993).

No que se refere à manutenção da integridade da mucosa intestinal e da função imune da mucosa, Beck & Wallace (1997) destacam que estudos com modelos animais têm evidenciado a importância da citocina IL-10, uma citocina anti-inflamatória produzida principalmente por células Th2, mas também por Th1 e monócitos. Dentre suas numerosas ações, destaca-se para IL-10 na DII, inibição da apresentação de antígenos, e subseqüente liberação de citocinas pró-inflamatória, e inibição da infiltração de neutrófilos e macrófagos nos sítios de injúria tissular, possuindo efeitos inibitórios sobre a quimiotaxia de macrófagos (Owczarek, et al. 2009).

Avanços no conhecimento da imunologia da DII têm levado a novos conceitos terapêuticos que alvejam os diversos aspectos do processo inflamatório desta enfermidade (Baumgart & Sandberg, 2007). No entanto, segundo Podolsky (2002), o tratamento para DII deve começar pelo diagnóstico preciso, que depende do conjunto da história clínica, dos achados de exame físico, endoscópico, radiológico e histológico, assim como dos exames laboratoriais. O resultado desta investigação permite distinguir a DC da RU, o que não é possível ocorrer em cerca de 10% dos pacientes.

A terapêutica convencional abrange o uso do ácido 5-aminosalicílico (5-ASA), de corticosteróides e de imunossupressores. O 5-ASA mantêm-se importante no tratamento da RU e DC. Atua bloqueando a produção de PGs e leucotrienos, inibindo a quimiotaxia de neutrófilos induzida por peptídeos bacterianos, seqüestrando espécies reativas de oxigênio e inibindo a ativação do NF-κB (Podolski, 2002; Biondo-Simões, et al., 2003). Os corticosteróides têm sido empregados inicialmente na remissão da doença, parecendo controlá-la de maneira complexa, o que pode incluir a modulação da fosfolipase A2, IL-1, TNF-α (Bauditz et al., 2002; Albuquerque, 2010), e

moléculas de aderência aos leucócitos. Agentes imunossupressores como a azatioprina e seu metabólito ativo, 6-mercaptopurina, têm sido extensivamente utilizados, apesar de seus efeitos colaterais, como o aumento do risco de linfoma. O mecanismo de ação desta droga permanece desconhecido, mas pode incluir supressão da geração específica de células T (Biondo-Simões, et al., 2003).

Na terapêutica não-convencional para a DII, o uso de anticorpos anti-TNF-α têm sido particularmente efetivos no tratamento da DC, especificamente o infliximabe, adalimumabe e certolizumabe (Gomollón & López, 2008). A modulação de outras citocinas também tem sido investigada, como a da IL-10 (Baumgart & Sandborn, 2007). Outra abordagem terapêutica é o bloqueio de células T e de inibidores seletivos de moléculas de adesão (Baumgart et al.,2005). Um estudo multicêntrico recente mostrou que o anticorpo monoclonal para integrina α4β7, o MLN02, tem eficácia na indução da remissão em pacientes com RU (Feagan et al., 2005), sugerindo que MLN02 seria uma alternativa atraente para pacientes que não respondem ao 5-ASA ou corticóides.

3 Mediadores envolvidos em processos fisiológicos e patológicos

3.1 Prostaglandinas

As PGs derivadas do ácido araquidônico são denominadas de PGs da série 2, sendo elas a prostaglandina E_2 (PGE_2), prostaglandina D_2 (PGD_2), prostaglandina I_2 (PGI_2), prostaglandina F_2 ($PGF_{2\alpha}$) e tromboxano A_2 (TXA_2) (Dey, Lejeune & Chadee, 2006). A hidrólise de fosfolipídios da membrana, mediados pela fosfolipase A_2, liberando ácido araquidônico no citoplasma, é a via biossintética comum para a produção destas PGs. Depois de liberado, o ácido araquidônico é convertido em intermediários endoperóxidos, PGG_2 e PGH_2, através da ação das enzimas ciclooxigenase (COX). As isoformas COX-1, constitutiva, e a induzida, COX-2, são as mais importantes (Smith et al., 1996).

Estudos mostram que a clássica hipótese de que prostanóides elaborados por COX-1 estão envolvidos em processos fisiológicos, e que a indução da expressão de COX-2 ocorre apenas em resposta a lesão tecidual, mediando processos de dor e inflamação, é muito simplista. Diferentes observações dos mecanismos de defesa da mucosa gástrica indicam que COX-2 desempenha um

importante papel regulador. Peskar (2005) destaca o papel de COX-2 na cura da úlcera, enquanto que sua inibição retarda o processo de cura. Por sua vez, Motilva et al. (2005) confirmaram o papel fundamental de COX-2 na cura de úlcera gástrica experimental aguda e crônica, tendo PGE_2 como principal produto.

A PGE_2 é considerada a mais importante prostaglandina para a fisiologia do TGI. Atuando sobre os receptores EP de membrana plasmática, subtipos EP_1, EP_2, EP_3, EP_4, acoplados à proteína G, desencadeia vias de sinalização que elevam os níveis de Ca^{2+} e AMP cíclico. O papel fisiológico de PGE_2 inclui estimulação fisiológica da liberação do muco, atuando sobre receptores EP_4 (em humanos e em ratos) e induzindo a transcrição do gene da mucina (Cho et al., 2005). O estímulo sobre EP_1 em ratos estimula a secreção de bicarbonato (Takeuchi et al.,1999a). Quanto à secreção ácida em ratos, sua ligação à EP_3 inibe, enquanto que à EP_4 estimula a secreção através da liberação de histamina (Kato et al., 2005). Também auxilia na manutenção do fluxo sangüíneo da mucosa (Gracioso et al., 2002; Abdel-Salam et al., 2001).

Apesar dos classicamente reconhecidos efeitos protetores de PGs, citados antes, o metabolismo do araquidonato está aumentado sob certas condições patológicas, incluindo infecção ou inflamação (Martel-Pelletier, Pelletier, & Fahmi, 2003), sendo bem documentado que este aumento é devido à COX-2. Experimentos *in vitro* revelaram que COX-2 foi induzida em células inflamatórias, como macrófagos e fibroblastos, em resposta a citocinas e fatores de crescimento (Xie et al., 1991). Experimentos *in vivo* também mostraram indução de COX-2 em células inflamatórias e nos locais de inflamação (Seibert et al.,1994), como em estudos de colite experimental (Camacho-Barquero, et al., 2007; Sánchez-Fidalgo et al., 2007; Talero et al., 2008).

Níveis de PGE_2 estão significativamente aumentados durante a DII. Um interessante estudo mostrou padrões definidos e identificou grandes diferenças na expressão de receptores EP_4 entre células cólicas humanas normais da lâmina própria e aquelas do cólon humano inflamado (Cosme et al., 2000). Na colite ulcerativa, além do aumento da expressão de receptores EP_4 em linfócitos T da lâmina própria, também foi demonstrado aumento dos níveis de EP_2 e EP_3 nas células epiteliais (Takafuji et al., 2000). Tomados em conjunto, estudos que investigam o papel do receptor EP_4 na colite destacam a importância dos eventos de sinalização que ocorrem nos tipos celulares da mucosa cólica a partir do acoplamento PGE2-EP4 como fundamentais na determinação desta doença inflamatória (Dey, Lejeune & Chadee, 2006).

3.2 Oxido nítrico (NO)

O NO é um radical livre que atua como modulador endógeno das funções do TGI, regulando a manutenção do tônus da musculatura lisa, peristaltismo intestinal, esvaziamento gástrico, produção de bicarbonato, manutenção do fluxo sangüíneo, secreção de muco e citoproteção adaptativa, juntamente com as PGs (Martín, Jiménez & Motilva, 2001). No entanto, estudos têm sugerido que o NO também pode ser um mediador crítico de várias desordens intestinais (Wallace, Mark & Miller, 2000). Este paradoxo pode ser explicado, pelo menos em parte, pela habilidade de diferentes concentrações produzirem efeitos opostos num mesmo tecido.

A formação do NO é catalisada, a partir do aminoácido arginina, pela enzima óxido nítrico sintase (NOS), que ocorre em pelos menos três isoformas, das quais e-NOS (*endothelial nitric oxide synthase*) e n-NOS (*neuronal nitric oxide synthase*) são constitutivas, enquanto a i-NOS (*inducible nitric oxide synthase*) é induzida (Holzer, 2001). NO sinaliza através da via da guanilato ciclase/GMP cíclico. Enquanto o NO produzido em baixas doses por n-NOS e e-NOS regula a homeostase, e beneficia a mucosa gastrintestinal, excessivas quantidades deste mensageiro, formadas por i-NOS, funcionam como substância citotóxica, lesionando os tecidos e afetando o sistema imune (Wallace, Mark & Miller, 2000).

A enzima i-NOS está localizada em macrófagos, hepatócitos, musculatura vascular lisa, neutrófilos e em células endoteliais. Sua expressão é regulada por fatores de transcrição de genes envolvidos na inflamação e na função imune, como o NF-κB (Neurath et al., 1996) que possui atividade nas células epiteliais das criptas intestinais, em macrófagos e em células epiteliais próximas aos pequenos vasos da mucosa inflamada (Roger et al, 1997).

O NO produzido pela isoforma i-NOS é essencial em processos inflamatórios, reparo de tecidos e mecanismos específicos de defesa do hospedeiro. Em macrófagos NO produzido por i-NOS contribui para a atividade antimicrobiana destas células, inibindo enzimas fundamentais para a atividade dos microorganismos. Também reage com ânion radical superóxido ($O_2^{\cdot}$), formando peroxinitritos ($ONOO^{\cdot}$) e outros compostos como trióxido de dinitrogênio (N_2O_3), os quais são altamente lesivos para o organismo (Martín, Jiménez & Motilva, 2001). A enfermidade que talvez possua mais dados sugerindo o papel do NO na sua patologia é a DII, na qual tanto os modelos experimentais como o humano mostram consideráveis evidências do aumento da produção de NO como resultado da ação de i-NOS (Zhang,et al., 1998).

4 Compostos polifenólicos na terapêutica

Os flavonóides compreendem um grupo de metabólitos secundários de plantas caracterizados pela estrutura química 2-fenilbenzopirano (Aron & Kennedy, 2008). Sua estrutura química geral baseia-se num esqueleto de 15 carbonos (C_6-C_3-C_6). Possui um anel (anéis A e C na figura 1), núcleo benzopirano (Havsteen, 2002), que, por sua vez, se liga ao segundo anel aromático nas posições C-2, C-3 ou C-4. O anel heterocíclico benzopirano (anel C), se funde ao anel aromático (anel A), e ao constituinte fenol (anel B). A posição da ligação do anel cromano determina a classe do benzopirano: 2-fenilbenzopirano compreende os flavonóides, 3-fenilbenzopirano, os isoflavonóides e 4-fenilbenzopirano, os neoflavonóides. Os três grupos têm a chalcona como precursor (Aron & Kennedy, 2008).

Levando-se em consideração a natureza química da molécula e a posição dos substituintes nos anéis A, B e C, os flavonóides são divididos em 14 grupos diferentes, sendo 7 destes particularmente bem conhecidos: flavonas, flavonóis, flavanonas, antocianidinas, chalconas, auronas e flavanois, ao qual pertence a maior e mais diversa classe de flavonóides monoméricos, os flavan-3-ois, que compreendem as unidades constitutivas dos taninos condensados (proantocianidinas) (Havsteen, 2001; Bravo, 1998, Dryden, Song & Mc Clain, 2006).

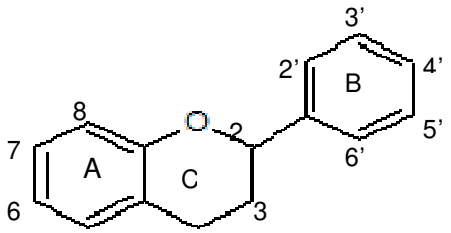
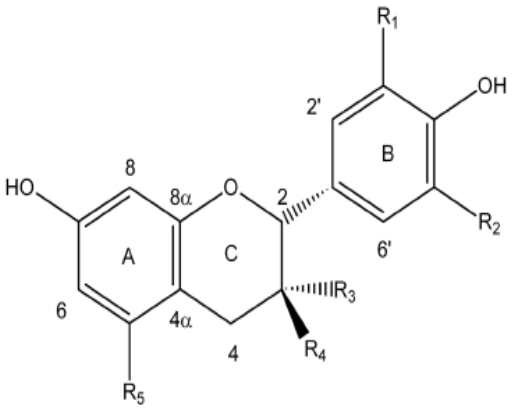


Figura 1- Estrutura básica dos flavonóides – 2-fenilbenzopirano.

Flavan-3-ois (catequinas) são o grupo de flavonóides mais comum na dieta, sendo considerados componentes funcionais de frutas, grãos, plantas medicinais e de suplementos dietéticos. Dentre os benefícios à saúde que lhes têm sido atribuídos, destacam-se sua ação antioxidante, anticancerígena, cardioprotetora, antimicrobiana, anti-viral, antiinflamatória, antimicrobiana e anti-ulcerogênica (Clarke & Mullin, 2008)

A figura 2 mostra exemplos de monômeros flavan-3-ol. A estereoquímica usual dos flavan-3-ois no carbono 2 é R A configuração 2S é menos comum e carrega o prefixo *enantio*, grafado como *ent*.



Monômeros 2R	R_1	R_2	R_3	R_4	R_5
(+)-afzelequina	H	H	H	OH	OH
(−)-epiafzelequina	H	H	OH	H	OH
(+)-catequina	H	OH	H	OH	OH
(−)-epicatequina	H	OH	OH	H	OH
(+)-galocatequina	OH	OH	H	OH	OH
(−)-epigalocatequina	OH	OH	OH	H	OH
(+)-fisetinidol	H	OH	H	OH	H
(−)-epifisetinidol	H	OH	OH	H	H
(+)-robinetinidol	OH	OH	H	OH	H

Figura 2 -. Exemplos de monômeros Flavan-3-ol. Muitos dos flavan-3-ol naturais são isômeros 2R.
Fonte: Aron & Kennedy, 2008.

Apesar de muitas das ações das catequinas terem sido identificadas, seus mecanismos de ação ainda não foram completamente elucidados. Sua ação mais bem fundamentada é antioxidante de doadora de hidrogênios e seqüestradora de radicais livres (Heijnen et al., 2001). Catequinas, particularmente epigalocatequina galato, são efetivas seqüestradoras de radicias livres *in vitro,* entretanto, alguns pesquisadores sugerem que estes compostos podem ter papel menos importante *in vivo*, devido aos baixos níveis circulantes e rápido metabolismo (Williams, Spencer & Rice-Evans, 2004), o que tem levado a investigação de catequinas em vias de sinalização celular. Pesquisas têm demonstrado que catequinas podem interagir seletivamente com a via de sinalização da *mitogen-activated protein kinase* (MAP kinase) (Kobuchi et al., 1999 & Kong, Yu and Chen, 2000), por exemplo, impedindo a ativação de c-Jun N terminal kinase (JNK), possivelmente por influenciar uma das muitas proteínas MAPKKK *upstream* que transduzem o sinal para JNK (Williams, Spencer & Rice-Evans, 2004).

A ingestão de catequinas tem sido associada com uma grande variedade de efeitos benéficos à saúde em estudos *in vitro, in vivo* e em ensaios clínicos. Seu grande potencial terapêutico associado a uma produção de baixo custo torna estes polifenois atrativos candidatos ao

tratamento de doenças relacionadas ao estilo de vida, como doenças cardiovasculares e DII. (Sutherland et al., 2006).

Outros compostos polifenólicos bastante conhecidos são os taninos, um grupo diverso formado como metabólitos secundários em plantas, incluindo um grande número de polifenois oligoméricos e poliméricos. Taninos condensados (proantocianidinas- PA) e hidrolisáveis (galotaninos e elagitaninos) são os taninos de ocorrência mais frequente (Frazier et al, 2010); e estão presentes na maioria das plantas, podendo variar de concentração nos tecidos vegetais, dependendo da idade e do tamanho da planta, da parte coletada, da época ou, ainda, do local de coleta (Teixeira et al., 1990; Simon et al.,1999).

PA existem como oligômeros (solúveis em água) contendo de 2 a 10 ou mais unidades de catequinas, e polímeros (insolúveis em água). As que contêm uma única ligação interflavan são classificadas como do tipo B e são ligadas, principalmente, através de ligações C4-C8 ou C4-C6 (Khanbabaee & van Ree, 2001) (Figura 3). Alternativamente, elas podem ser esterificadas por grupos ligados à hidroxila do C3, contendo duas ligações interflavan e uma ligação éter adicional entre C2 e O7, e são classificadas como proanticianidinas do tipo A (Rodrigues et al.,2007).

Procianidinas (R=H) e prodelfinidinas (R=OH) são os tipos mais comumente encontrados de PA condensadas. Este é o tipo de taninos mais frequentemente distribuído no reino vegetal; estão associadas a plantas com hábito de crescimento arbustivo (Haslam, 2007). Quantidades significativas de PA são consumidas em alimentos como frutas, cereais e grãos, como também em bebidas, incluindo chás, achocolatados e vinho tinto (Benavides et al., 2006).

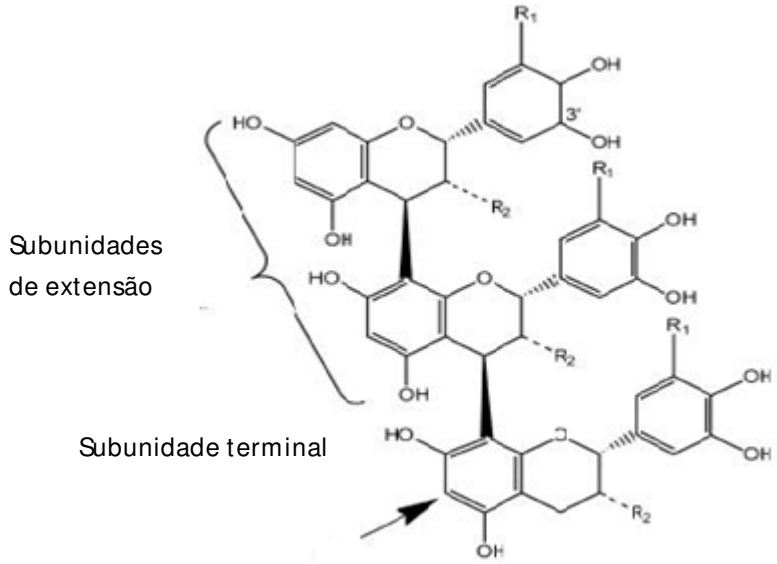


Figura 3 - Proantocianidina do tipo B.
Fonte: Aron & Kennedy, 2008

R_1= H ou OH, R_2=OH ou galoil

Acredita-se que os taninos exercem seus efeitos terapêuticos na saúde humana, pelo menos em parte, em virtude de três características gerais que são comuns em maior ou menor grau, aos dois grupos de taninos: a) complexação com íons metálicos (ferro, manganês, alumínio, cálcio, entre outros); b) atividade antioxidante e seqüestradora de radicais livres e, c) habilidade de se complexar com outras moléculas como proteínas e polissacarídeos (Haslam, 1996). Esta última característica é particularmente importante no processo de cura de feridas, queimaduras e inflamação, através da formação de uma camada protetora sobre a pele, complexo tanino/proteína e/ou polissacarídeo sobre a pele ou mucosa danificada. Debaixo dessa camada, o processo natural de cura, pode, então, ocorrer. Um processo similar ocorre em casos de úlcera gástrica em que a camada tanino/proteína complexados protege a mucosa do estômago (Haslam, 1989). Alguns grupos de taninos também agem no metabolismo do ácido araquidônico, em leucócitos, exercendo um importante papel na reversão da inflamação, sendo usados em tratamentos que promovem a cura de feridas (Okuda, 2005).

5 Espécie estudada

Abarema cochliacarpos (Gomes) Barneby & Grimes é uma espécie endêmica do Brasil. Árvore ou arbusto de 1-30m, com frutos contorcidos, sementes brancas e cinzas e flores com estames alvos. *A.cochliacarpos* tem distribuição na Mata Atlântica, entre manchas de cerrado em contato com a caatinga. Estava incluída entre as espécies ameaçadas de extinção pelo World Conservation Monitoring Centre (1998), mas de acordo com Iganci & Morim (2009), o atual conhecimento da distribuição geográfica da espécie sugere que esta classificação seja reavaliada, permitindo classificá-la atualmente como fora de perigo.

O Gênero *Abarema* (Leguminosae, Mimosaceae) compreende 49 espécies neotropicais (Lewis & Rico Arce 2005) e, para a flora brasileira, são citadas 30 espécies com centros de diversidade nas Florestas Amazônica e Atlântica (Barneby & Grimes 1996; Iganci & Morim 2009a), sendo amplamente distribuída no Nordeste e no Sudeste do Brasil, abrangendo os estados do Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Espírito Santo, Bahia, Minas Gerais, Rio de Janeiro e São Paulo (Iganci & Morim, 2009 b).

Abarema cochliacarpos é amplamente utilizada *in natura* no Brasil como planta medicinal (Migliatti, 2003). O interesse pelo estudo desta espécie partiu de um levantamento

etnofarmacológico de plantas medicinais utilizadas popularmente contra distúrbios gastrintestinais no Povoado Colônia Treze, município de Lagarto, Estado de Sergipe, inserido na região do semi-árido do Nordeste do Brasil, no bioma Caatinga (Silva, 2003). Na comunidade do Povoado Colônia Treze a decocção da casca (Figura 4) é utilizada contra inflamação, úlcera gástrica e para lavar úlceras externas e inflamação genital feminina. Registrou-se também nesta comunidade o uso de *A. cochliacarpos* em preparações caseiras denominadas garrafadas, nas quais a casca é macerada em vinho branco ou cachaça, produzindo-se assim uma tintura que é utilizada contra úlcera gástrica e inflamação (Silva, 2003).

Usos similares foram mostrados por outros pesquisadores (Agra et al., 2008) e outro trabalho recente destaca o uso popular desta espécie contra inflamação, dor, cicatrização e câncer (Santos, 2008). Entretanto, poucos estudos têm sido realizados para avaliar a atividade farmacológica desta espécie. A capacidade de inibição do crescimento bacteriano *in vitro* do extrato hidroalcoólico da casca têm sido confirmado por alguns autores (Araújo et al., 2002; Santos et al., 2007) e, mais recentemente, os extratos aquoso e metanólico têm mostrado atividade antinociceptiva (Silva et al, 2009). Partindo-se deste estudo, testou-se o extrato aquoso bruto das cascas de *A. cochliacarpos* no modelo de úlcera gástrica induzida por etanol mostrando-se pela primeira vez que o extrato aquoso reduzia as lesões gástricas (Silva *et al*, 2006).

A)

B)

C)

Figura 4 - Tronco, flores e frutos de *Abarema cochliacarpos*. Fonte: (A) Foto da autora. (B) e (C) Disponíveis em www.flickr.com/photos.

II Objetivos

Foram objetivos deste trabalho investigar para *Abarema cochliacarpos* (Gomes) Barneby & Grimes:

- A atividade antiulcerogênica gástrica nos modelos de etanol e ácido acético, bem como determinar possíveis mecanismos de ação envolvidos nesta atividade.

- A atividade antiinflamatória intestinal da fração butanólica do extrato metanólico da espécie em modelos de colite ulcerativa aguda e crônica, induzidas por ácido trinitrobenzeno sulfônico (TNBS), bem como possíveis mecanismos de ação envolvidos com a a tividade.

III Material e Métodos

1 Coleta da espécie

Abarema cochliacarpos foi coletada no Povoado Colônia Treze, em Lagarto, Sergipe, no período de maio a julho de 2006. A coleta foi acompanhada por um praticante da medicina popular do povado. A identificação botânica foi feita pela Dra. Chistine Niezgoda do Field Museum of Natural History (Chicago, IL) A exsicata desta espécie está depositada no Herbário da Universidade Federal de Sergipe, sob o número 007628.

2 Preparação dos extratos brutos e de partições

As cascas de *A. cochliacarpos* (1200 g) foram submetidas à secagem em estufa (40 °C) e, em seguida, trituradas em moinho de facas. Este trabalho foi realizado no Laboratório de Farmacologia do Departamento de Fisiologia da Universidade Federal de Sergipe (UFS). O pó resultante foi enviado ao Laboratório de Fitoquímica do Instituto de Bioquímica da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araraquara, onde foram preparados os extratos brutos e partições, sob a orientação do Prof. Dr. Wagner Villegas.

A casca triturada da espécie foi exaustivamente submetida a solventes de polaridade crescente. Utilizando-se 3 litros do solvente clorofórmio ($CHCl_3$), o material foi macerado por 6 dias. Decorrido este tempo, material foi seco a temperatura ambiente para a adição de 5 litros do solvente metanol (CH_3OH), produzindo-se o extrato metanólico por percolação durante 15 dias, à temperatura ambiente. Os solventes foram evaporados a 40 °C, sob pressão reduzida, produzindo o extrato clorofórmico e o extrato metanólico brutos, com rendimentos de 0.40% (4.81g) e 34,61% (415,40 g), respectivamente.

Os melhores resultados apresentados pelo extrato metanólico no modelo de indução de úlcera gástrica levaram à sua partição. Este extrato foi submetido à partição líquido-líquido com solventes de polaridade crescente, obtendo assim frações semi-purificadas. Assim, 200 g deste extrato foram dissolvidos em 1300 mL de água destilada num funil de separação. Foram particionadas três vezes, adicionando-se 100 mL de acetato de etila em cada uma das vezes ao funil de separação. A fração acetato de etila (EAF) foi evaporada a 35°C sob pressão reduzida, obtendo-se um rendimento de 50.12 g. A fase aquosa foi particionada novamente, adicionando-se 100 mL de *n*-

butanol, por três vezes, produzindo 99.69 da resultante fração *n*-butanólica (AC) e 50.19 g de fração aquosa (AQF).

3 Triagem fitoquímica dos extratos

3.1 Espectrometria de Massas

A composição química da AC de *A. cochliacarpos* foi realizada por Espectrometria de Massas com ionização por *electrospray* (ESI/MS) e por Espectrometria de Massas acoplada à Espectrometria de Massas com ionização por electrospray MS/MS**.** O estudo foi realizado utilizando-se o Finnigan (Thermo Finnigan, San José, CA, USA) LCQ Deca íon trap instrument. Amostras da AC foram diluídas em MeOH/H_2O 1:1 v/v para a obtenção de concentrações de 0.1% e infundidas na fonte de ionização por ESI. Para análise dos compostos, o aparelho foi operado no modo negativo. Espectros foram escaneados na faixa de m/z 50 – 1000 no modo MS1 e de 60-340 e 50-1000 no modo MS/MS. Este estudo foi realizado no *Centro de Investigación, Tecnología y Innovación* (CITIUS) da Universidade de Sevilla, Espanha, sob a orientação de Maria Eugenia Soria Díaz.

3.2 Ressonância Magnética Nuclear (RMN)

Amostra da fração butanólica de *A. cochliacarpos* foram diluídas em D6 dimetil-sulfóxido (DMSO-d6) para a obtenção do espectro de RMN, utilizando-se espectrômetro Varian INOVA 500, operado at 125MHz para ^{13}C. Os deslocamentos químicos são dados como δ (ppm), tendo tetrametilsilano (TMS) como padrão interno. Este estudo foi realizado no Instituto de Química (IQ) da Universidade Estadual de Campinas –UNICAMP, sob a orientação da Profª Drª Anita Marsaioli.

4 Procedimentos Experimentais

4.1 Drogas utilizadas

As drogas utilizadas nos protocolos para estudo das atividades farmacológicas (e de seus mecanismo de ação) dos extratos e frações foram: Lansoprazol (MEDLEY, Campinas, Brasil), Tween 80 (Sigma, Chemical Co, St. Louis, USA), Carbenoxolona (Sigma, Chemical Co, St. Louis, USA), Ácido Acético P.A. (CHEMCO, Campinas, Brasil), Cloreto de Sódio P.A. (CHEMCO,

Campinas, Brasil), Etanol P. A. (Sigma-Chemical Co., St. Louis, MO, USA), ácido trinitrobenzenosulfônico- TNBS (Sigma-Chemical Co., St. Louis, MO, USA).

4.2 Animais

Para o estudo da atividade antiulcerogênica de *A. cochliacarpos* foram utilizados Ratos (*Rattus norvegicus*) machos, da linhagem Wistar (150-250 g) e para o estudo de toxicidade aguda camundongos (*Mus musculus*) machos e fêmeas, da linhagem Swiss provenientes do Centro de Bioterismo da Unicamp (CEMIB). Para o estudo da atividade antiinflamatória intestinal, realizado na Universidade de Sevilla, Espanha, utilizou-se ratos Wistar machos e fêmeas (180-200 g), provenientes do Biotério da Universidade de Sevilla. Os animais foram aclimatados às condições dos respectivos biotérios por um período de 20 dias antes dos experimentos, sob temperatura de 23±2ºC e ciclos de claro e escuro controlado de 12 horas. Os animais foram alimentados com ração industrial Nuvital (Nuvilab) e Panlab (Barcelona, Espanha) e água *ad libitum*. O período de jejum está de acordo com o utilizado para cada metodologia e especificado nos respectivos itens.

Os protocolos experimentais foram aprovados pela Comissão de Ética em Experimentação Animal - CEEA/IB/UNICAMP, seguindo as recomendações do *Canadian Council on Animal Care*, com o protocolo de número 1287-1, e pelo Comitê de Ética em Experimentação Animal da Universidade de Sevilla, seguindo as recomendações da *Directive of the European Counsel* 86/609/EC.

4.3 Toxicidade Aguda

Camundongos machos e fêmeas Swiss foram separados em grupos da seguinte forma: 1) Tween 80 a 8% 1mL.kg^{-1}, 2) fração butanólica do extrato metanólico de *A. cochliacarpos* 5000 mg.kg^{-1}. Após os tratamentos os animais foram observados durante 14 dias; no 14° dia os animais foram sacrificados e os órgãos (coração, fígado, rins e pulmão) removidos para pesagem (Souza Brito, 1994).

4.4 Estudo da Atividade Antiulcerogênica

4.4.1 Lesão gástrica aguda induzida por Etanol em Ratos

Empregou-se o método descrito por Morimoto et al. (1991). Ratos machos Wistar foram mantidos em jejum por 24 horas. Os grupos experimentais os extratos e/ ou frações de *A. cochliacarpos* nas doses de 100, 200 e 400 $mg.kg^{-1}$. O grupo controle positivo foi tratado com lansoprazol (30 $mg.kg^{-1}$), enquanto o grupo controle negativo recebeu salina (NaCl a 0,9%) ou Tween 80 a 8%, ambos em volume equivalente a 10 $mL.kg^{-1}$. Após 1 hora dos tratamentos, os animais receberam oralmente etanol absoluto (1mL/animal). Decorrida 1 hora da administração do álcool, os animais foram sacrificados e os estômagos foram abertos pela grande curvatura, lavados com salina para avaliação das lesões, permanecendo em formalina 5%. Os estômagos foram fotografados e a área de lesão foi calculada com auxílio do programa Bioview 4 (AvSoft, Brasil), um software de análise de imagens (Khan, 2004).

4.4.2 Lesao gástrica induzida por ácido acético

O experimento foi realizado segundo metodologia descrita por Takagi et al (1969). Ratos Wistar foram anestesiados com ketamina (50 $mg.kg^{-1}$) e xilazina (10 $mg.kg^{-1}$) para exposição do estômago através de uma incisão de aproximadamente dois centímetros, realizada abaixo da apófise xifóide. Em seguida, foram injetados, com auxílio de uma micro-seringa, 0,05 mL de uma solução de ácido acético 30 % na camada subserosa da junção do fundo com o antro do estômago. Dois dias após administração do ácido, foram iniciados os tratamentos, por via oral, com a AC (150 $mg.kg^{-1}$), de lansoprazol (30 $mg.kg^{-1}$) (controle positivo) e com 10 $mL.kg^{-1}$ de Tween 80 a 8%, os quais persistiram durante 14 dias consecutivos.

Ao final do tratamento, os animais em jejum de 12 horas, foram sacrificados e seus estômagos removidos, abertos no sentido da maior curvatura e os estômagos retirados e fotografados para determinação da área de lesão, conforme descrito anteriormente. Foram coletadas amostras de tecido para análises histológicas e imunohistoquímicas. Amostras de tecido da mucosa foram imediatamente congeladas em nitrogênio liquído para a realização de estudos bioquímicos.

4.4.3 Determinação da produção de muco

O muco da parede gástrica foi determinado, segundo Corne et al. (1974), em ratos machos Wistar submetidos à ligadura do piloro. Após 24 horas de jejum, os diferentes grupos experimentais foram tratados com AC (150 $mg.kg^{-1}$), Carbenoxolona (200 $mg.kg^{-1}$) (controle positivo) e Tween 80 a 8% como controle negativo, por via oral, em volume equivalente a 10 $mL.kg^{-1}$. Uma hora após receberem os tratamentos, os animais foram anestesiados com ketamina (50 $mg.kg^{-1}$) e xilazina (10 $mg.kg^{-1}$) e após, sofrerem uma incisão longitudinal logo abaixo da apófise xifóide para a localização e amarradura do piloro; em seguida, as incisões foram suturadas. Quatro horas após cirurgia, os animais foram sacrificados, as incisões reabertas, os estômagos retirados e abertos no sentido da grande curvatura; os segmentos glandulares do estômago foram removidos e pesados. Cada segmento foi transferido imediatamente para um tubo contendo 10 mL de Alcian blue 0,1% (em solução de sacarose 0.16 M tamponada com acetato de sódio 0.05 M, pH 5). Após imersão por 2 h nesta solução, o excesso de corante dos estômagos foi removido por duas lavagens sucessivas com 10 mL de solução de sacarose 0.25 M, primeiro por 15 min e, depois, por 45 min. O corante complexado ao muco da parede gástrica foi extraído com 10 mL $MgCl_2$ 0.5 M por agitação intermitente por 1 min, a intervalos de 30 min, durante 2 h. A 4 mL da mistura foram adicionados 4 mL de éter etílico e, então, a solução foi submetida à agitação por 2 minutos. A emulsão obtida foi centrifugada por 10 minutos a 2000×*g* e o precipitado foi descartado. As absorbâncias foram lidas em espectrofotômetro a 598 nm. A leitura foi feita após realização de uma curva padrão, com várias concentrações de Alcian blue. Os resultados foram expressos em μg de Alcian blue/mL/g de tecido.

4.4.4 Avaliação dos parâmetros bioquímicos da secreção gástrica

De acordo com Shay et al. (1945), os animais, após 24 horas de jejum e sob anestesia (ketamina 30 mg/Kg e xilazina 0,3 mg/Kg), sofreram uma incisão longitudinal logo abaixo da apófise xifóide para localização e amarradura do piloro. AC 150, Lansoprazol (30 $mg.kg^{-1}$), controle positivo e Tween 80 a 8%, controle negativo, num volume equivalente a 10 $mL.kg^{-1}$, foram administrados por via intraduodenal logo após a ligadura do piloro. Em seguida, as incisões foram suturadas. Quatro horas após a cirurgia, os animais foram sacrificados, as incisões reabertas e os estômagos retirados. O conteúdo estomacal foi coletado para determinação do volume gástrico e pH

da secreção gástrica (utilizando um peagâmetro modelo Q 400ª Quimis Aparelhos Científicos Ltda – Brasil).

4.4.5 Estudo histológico

O estudo histológico das amostras provenientes do modelo de indução de úlcera gástrica por ácido acético foi realizado no Departamento de Morfologia, Laboratório de Histologia da Universidade Estadual Paulista (UNESP- Botucatu/SP), sob orientação da Prof. Drª Claudia Helena Pelizzon. As amostras foram fixadas em placas de isopor com alfinetes e imersas numa solução de ALFAC (formalina, álcool 80%, ácido acético) por 24 horas. As peças foram desidratadas e incluídas em paraplast. Posteriormente, os blocos de paraplast foram cortados (7 μm de espessura) em micrótomo de maneira semi-seriada. As lâminas obtidas, segundo esse processo foram submetidas à coloração por Hematoxilina-Eosina (HE) (Behmer et al., 1976) e ou ácido periódico de Schiff (PAS) (Vacca, 1985) para análises histológicas em microscopia de luz.

4.4.6 Imunohistoquímica

Uma lâmina representativa de cada grupo de animais submetidos ao método de indução de úlcera por ácido acético foi levada ao Departamento de Morfologia, Laboratório de Histologia da Universidade Estadual Paulista (UNESP- Botucatu/SP) para estudo de antígenos tissulares. As amostras foram testadas para os anticorpos HSP-70, PCNA, VEGF, COX-2 e VIP. Após desparafinização, bloqueou-se a peroxidase endógena com H_2O_2 a 3% por 10 minutos. Em seguida, recuperou-se a antigenicidade com tampão Citrato (pH 6,0) e microondas. As amostras foram lavadas em PBS (tampão fosfato 0,01M, pH 7,4), uma vez por 5 minutos e foi bloqueado o sítio inespecífico com leite desnatado sem Ca^{+2} a 1% por 10 minutos. Em seguida, as seções foram incubadas *overnight* com os anticorpos primário monoclonal *mouse* para PCNA (Novo Castra NCL-PCNA) (1:100), COX-2, Cayman Chemical) (1:200), marcador de vasos (VEGF, Novo Castra) (1:50), HSP-70 (Santa Cruz Biotechnology SC-1060) (1:100) e vasoactive intestinal peptide - VIP (Novo Castra) (1:100).

Após lavagem das amostras por duas vezes com PBS (0.01 mol/l PBS, pH 7.4), durante 5 minutos, incubou-se o anticorpo secundário por 30 minutos, lavou-se com PBS e incubou-se com o Complexo Steptavidina-biotina-Peroxidase. A revelaçao foi feita com 3-3' Diaminobenzidina tetrahydrochloride (DAB, Sigma) em 20 mL de PBS e 6μL H_2O_2 30%; as amostras foram contra-

coradas com HE e analisou-se ao microscópio (LEICA DM) acoplado com o software de captura de imagens (Leica QWin Standard Versão 3.1.0, Reino Unido).

4.5 Estudo da Atividade Antiinflamatória Intestinal

A colite induzida por TNBS foi o modelo utilizado como descrito por Morris et.al. (1989). Nele a atividade antiinflamatória intestinal de *Abarema cochliacarpos* foi investigada. Ratos, após jejum de 12 horas, foram anestesiados com hidrato de cloral 12% (Panreac, Barcelona, Espanha) por via intraperitoneal. Ácido trinitrobenzenosulfônico-TNBS (Sigma-Chemical Co., St. Louis, MO, USA) foi administrado, por via retal, através de uma cânula de poliuretano (diâmetro externo de 2 mm) inserida no ânus, avançando até 8 cm da região proximal. TNBS, dissolvido em etanol (50%, v/v), foi instilado dentro do colon através de uma cânula (TNBS-10 mg ou TNBS-30 mg em 0.25 mL de etanol para induzir colite aguda ou crônica, respectivamente). Utilizou-se o grupo Sham como controle de referência, o qual recebeu salina fisiológica ao invés de TNBS em volume comparável. Animais do grupo Sham e do grupo TNBS também receberam o veículo (2% Tween 80 em volume equivalente a 10 mL/kg) via oral.

Depois da instilação de TNBS, os animais foram mantidos de cabeça para baixo por 2 a 3 minutos para impedir a perda de TNBS. A fração butanólica de *A. cochliacarpos* (100mg.kg^{-1} e 150 mg.kg^{-1}) foi suspensa em 2% Tween 80 (Sigma-Chemical Co., St. Louis, MO, USA) (Santos el al, 2004) e foi administrada num volume equivalente a 10 mL/kg por via oral 48, 24 e 1 h antes da indução de colite e também 24 h depois. Os animais foram sacrificados 48 horas depois da indução, utilizando-se hidrato de cloral 24%. Após sacrifício dos animais, foi retirado o cólon em sua totalidade e procedida a limpeza dos distintos segmentos intestinais, removendo-se os restos de tecido adiposo e adesões mesentéricas, sobre uma placa de Petri com gelo. O cólon foi lavado com solução salina isotônica e foi determinado seu comprimento sobre uma tensão constante de 2 g, bem como seu peso. O tecido foi aberto longitudinalmente, removido 10 cm do colon distal e fotografado para avaliação de lesão macroscópica. Para estudos histológicos, foram obtidas amostras do cólon da área proximal imediatamente adjacente à lesão. O cólon foi dividido em vários fragmentos longitudinais que foram imediatamente congelados em nitrogênio líquido para determinação dos parâmetros bioquímicos. A área de lesão foi quantificada como descrita no item 4.4.1. A presença de adesões (score 0–2), e/ou consistência das fezes (score 0–1) foi avaliada de acordo com o critério de Bobin-Dubigeon et al. (2001), com algumas modificações.

4.5.1 Mecanismos envolvidos com a atividade antiinflamatória

a) Avaliação da Infiltraçao de neutrófilos

A atividade da MPO, como indicador de infiltração de neutrófilos foi avaliada de acordo com Bradley et al. (1982). Foram obtidas amostras de todos os animais e rapidamente colocadas em salina gelada e guardadas a -80°C. O tecido foi pesado e homogeneizado com 10 volumes de tampão fosfato de sódio (50 mM, pH 7.4). O homogenato foi centrifugado a 20,000×*g*, 20 min, 4 °C. O precipitado foi novamente homogeneizado em 10 volumes de tampão fosfato de sódio, pH 6.0, contendo 0.5% de hexadecil-trimetilamonio bromidio (HETAB) e 10 mM ácido etilenodiamino tetraacético (EDTA). Este homogenato foi submetido a um ciclo de congelamento/descongelamento e a um breve período de sonicação sob ultrassom. O sobrenadante (50µL) foi diluído em 10 volumes de 50mM PBS, pH 6. Então foi adicionado consecutivamente 50µL de Orto-dianisidina (0,067%), 50µL de 5% de HETAB e 50µL de peróxido de hidrogênio (0.003%). Os componentes da reação foram incubados à temperatura ambiente por 5 minutos. A mudança na absorbância a 450nm foi medida com leitor de placas (Labsystem Multiskan EX, Helsinki, Finlândia). Uma unidade de MPO foi definida como a quantidade de enzima presente que produz uma mudança na absorbância de 1.0 U/min no volume de reação final.

b) Determinacão dos níveis colônicos de TNF-α e IL-l0

Amostras do cólon distal foram pesadas e homogeneizadas, em 10 volumes de tampão fosfato salina (PBS, pH 7.2) e 1% de soro albumina bovina (BSA) a 4 °C; logo após as amostras foram centrifugadas a 12,000 x g for 10 minutos e o sobrenadante foi coletado e estocado a -80°C. Níveis de citocinas da mucosa foram medidos com kits quantitativos de ensaio imunoenzimático, Enzyme Linked-Immuno-Sorbent Assay (ELISA) para TNF-α (eBioscience, San Diego,CA) e IL-10 (Diaclone, Besançon,France). Valores de TNF-α e IL-10 foram expressos como pg/mg tecido.

c) Western blotting

Amostras de tecido cólico foram pesadas e homogeneizadas em solução tampão (Tris–HCl 50 mM, pH 7.5, $MgCl_2$ 8mM, ácido etileno glicol-bis b-aminoetileter N,N,N,N-tetraacético (EGTA) 5mM, EDTA 0.5 mM, leupeptina 0.01 mg/ml, pepstatina 0.01 mg/ml, aprotinina 0.01 mg/ml,

fenilmetilsulfonilflúor (PMSF) 1 mM, e NaCl 250 mM, centrifugadas a 12000 x g, 4 ºC, por 15 minutos. Alíquotas do sobrenadante foram coletadas e estocadas a -80 °C. A concentração de proteínas foi quantificada pelo método de Bradford (1976). Valores determinados de proteína (50 µg) foram aplicados em gel de poliacrilamida com dodecilsulfato de sódio (SDS-PAGE) e submetidos à eletroforese, com solução tampão (Trisma base 25 mM, glicina 1,92 mM, SDS 1%). O SDS-PAGE foi submetido a 110V, inicialmente, até a passagem da linha demarcada pela fase de empilhamento das proteínas (*stacking*) e 150V até o final do gel de corrida (*running*). A seguir, as proteínasm, separadas no SDS-PAGE, foram transferidas para uma membrana de nitrocelulose, em equipamento de eletrotransferência, com as membranas embebidas em solução tampão de transferência (Trisma base 25 mM, glicina 192 mM, metanol 20%), mantidas em voltagem constante de 100V por 2 horas. As membranas de nitrocelulose, contendo as proteínas transferidas, foram incubadas em solução bloqueadora (leite em pó desnatado a 5%) por uma hora, a fim de diminuir ligação inespecífica de proteínas. Em seguida, as membranas foram submetidas a lavagens com tampão básico (NaCl 150 mM, Trisma base 10 mM, Tween 20 a 0.05%), em intervalos de 10 minutos. A membrana foi incubada a 4 °C, durante uma noite, usando anticorpo específico contra i-NOS (Santa Cruz Biotechnology) e COX-2 (Santa Cruz Biotechnology) a diluição de 1:3000, JNK1/3 (Santa Cruz Biotechnology) e pJUNK (Santa Cruz Biotechnology). Na manhã seguinte, a membrana de nitrocelulose foi lavada em tampão básico por 40 minutos e, em seguida, incubada à temperatura ambiente, por 1h, com o anticorpo Imunoglobulina G *anti-rabbit* (Pierce Biotechnology, IL, U.S.A). Para verificar se a mesma quantidade de proteína foi carregada, analisou-se a expressao da β-actina, usando-se o anticorpo anti-β-actina (Dako, Dinamarca). A detecção das bandas imuno-reativas foi feita utilizando-se uma solução de quimioluminescência (SuperSignal® West Femto Chemiluminescent Substrate, Pierce, IL, USA). As densidades das bandas das amostras foram capturadas através do equipamento FUJIFILM LAS-3000 mini, através do *software* LAS 3000. Os dados densitométricos foram analisados pelo programa KODAK 1D, Image Analysis Software).

d) Estudo histológico

O estudo das amostras do modelo de colite ulcerativa foi realizado na Universidade de Sevilla, Espanha. Utilizou-se amostras de tecido do cólon inflamado distal de cada animal, fixadas em formaldeído 4%, em seguido submergido em sacarose 20-30% e depois embebidas em Tissue-

Tek® O.C.T™ (Tissue-Tek Sakura, Netherlands). Foram feitos cortes de 7 μm do tecido a -25°C com o criótomo MICROM HM 525 (Thermo Fisher Scientific, Waltham, MA). Os fragmentos foram montados em lâminas e as seções foram limpas, hidratadas e coradas com hematoxilina e eosina, para avaliação histológica da lesão colônica, de acordo com protocolo padrão. Todas as seções de tecido foram examinadas em microscopia de luz com o microscópio Olympus BH-2 (GMI, MN, USA) e após, as imagens capturadas foram analisadas com o software Motic Images 2000.

5 Análise estatística

Todos os resultados foram expressos como média ± erro padrão da média. A significância estatística das diferenças entre as médias foi determinada por análise de variância (ANOVA) uma via, seguido do teste de Tukey. O limiar de significância foi estabelecido para $p<0.05$. O programa estatístico utilizado foi o Grafpad Prisma 5.

IV Resultados e Discussão

Todos os dados obtidos nesse trabalho foram organizados na forma de artigo (submetido, publicado e em preparação)

Artigo 1 - Artigo submetido ao *Journal of Ethnopharmacology*

Título: *Abarema cochliacarpos*: Gastroprotective and Ulcer Healing Activities

1 - Introduction

Phenolic compounds, which include tannins and flavonoids, are apparently related to the interesting anti-inflammatory, wound healing, antioxidant and antiulcerogenic properties assigned to several medicinal plants, such as Brazilian Cerrado biome species that had its pharmacological proprieties investigated by our group (Moleiro et al., 2009; Sannomya et al., 2004; Kushima et al., 2009). On the other hand, some studies have indicated that phenolic compounds of the Caatinga plants appear in essentially 100% of some groups of medicinal plants in the region (Almeida et al., 2005).

Catechins (flavan-3-ols), such as catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate, are particularly abundant in steam bark of the species of Mimosaceae family (Santos et al., 2002). The antioxidant and free radical scavenging properties are the most renowned catechins' biological action (Sutherland et al., 2005). However, their actions on alternate cellular pathways, protect against cytotoxicity, anti-inflammatory actions and anticholesterolemia effects, have been investigated for many research (Heijnen et al., 2001,Williams, Spencer & Rice-Evans, 2004).

Tannins are potent scavengers of peroxyl radicals and can also interact with mucus proteins, improving their cytoprotective effect by forming a protein lining over the gastrointestinal mucosa (Okuda, 2005, Moleiro et al., 2009). They are used in treatments fostering wound. Araújo et al. (2008) studied plants with elevated levels of phenolic compounds within specific groups of plants with popular therapeutic uses cited above. They concluded that Caatinga medicinal plants which are known and/or used for their wound-healing or anti-inflammatory properties tend to have high tannin contents. Moreover, these compounds appear in elevated concentrations in some species that are

intensively used by the local communities and correlate with the therapeutic indications attributed to them (Monteiro et al., 2006), like the *Abarema cochliacarpos*.

Abarema cochliacarpos is a Brazilian native plant occurring mainly in the Atlantic Forest and in the Caatinga biomes. It is a tree species of the legume family Mimosaceae (IUCN, 2009), which is popularly known as "barbatimão". An ethnopharmacological survey accomplished in a rural community in the Caatinga (dryland) area in Lagarto city, Sergipe, Northeastern Brazil by Silva et al. (2006) identified the popular use of the steam bark of the *Abarema cochliacarpos* (Gomes) Barneby & Grimes. In this community, the decoction of the steam bark is used wash external ulcers and the ingestion of this decoction or the tincture, which is made by placing the bark in white wine or "cachaça", used against inflammation and gastric ulcers, among other uses (Silva, 2003 and Silva et al.,2006). Other authors also observed a similar use in different traditional communities (Agra et al., 2008, Santos, 2008).

Regarding its chemical composition, the butanolic fraction of *A. cochliacarpos* was characterized by electrospray ionization (ESI)/mass spectrometry (MS) and ESI/MS/MS as having high content of polyphenols. The tannins belong to a condensed subgroup, the proanthocyanidins, and the major constituents are catechins and the minor are the dimers and trimers. The nuclear magnetic resonance spectroscopy (NMR) characterization shows the major constituent are (+)-catechins (da Silva et al., 2010).

Peptic ulcer is one of the major gastro-intestinal diseases and it is caused by multiple factors, including stress, smoking, nutritional deficiencies, noxious agents such as alcohol, nonsteroidal anti-inflammatory drugs (NSAIDs), *Helicobacter pylori* infection, among others (Belaiche et al., 2002). Historically, our understanding of the pathophysiology of peptic ulcer disease focused on abnormalities in the secretion of gastric acid and pepsin, and on the suppression of acid (e.g. H_2 receptor antagonists, proton-pump inhibitors) as a treatment strategy (Yuan, Padol y Hunt, 2006). Although suppressors of acid secretion have been a mainstay to the promotion of ulcer healing for three decades, there is an increased interest in recent years in the mechanisms through which ulcers heal, and the possibility that both the speed and quality of healing may be pharmacologically modulated. Healing requires angiogenesis in the granulation tissue at the base of the ulcer, together with the replication of epithelial cells at the ulcer margins and subsequent re-establishment of glandular architecture (Wallace, 2008). Plant extracts are some of the most attractive sources of new drugs and have been shown to produce promising results in the treatment

of gastric ulcers (Borrelli & Izzo, 2000). In traditional medicine for example, several plants and herbs have been used to treat gastrointestinal disorders, including gastric ulcers (Calvo et al, 2007; Silva et al., 2009; Vasconcelos et al., 2008). This is an important reason to investigate antiulcer effect of medicinal plants with traditional use in gastric disease.

Concerning the pharmacologic effects, the hydroalcoholic extract stem bark of *Abarema cochliacarpos* showed antimicrobial activity (Araújo et al., 2002; Santos et al., 2007). Both crude aqueous and methanol extracts also showed antinociceptive effects (Silva et al., 2009). In our previous study, the antiulcer effect of the aqueous extract of the bark was tested in experimental alcohol gastric ulcers demonstrating for the first time a reduction of the lesions (Silva et al., 2006) and more recently, the butanolic fraction of the methanolic extract was tested on acute experimental trinitrobenzene sulfonic acid (TNBS) colitis model and showed antiinflammatory effects (Silva et al., 2010).

Considering the association of both anti-inflammatory and antiulcerogenic effects of the *Abarema cochliacarpos* extract and fraction, the present work was carried using two approaches. First, we have performed the pharmacological screening of the antiulcerogenic properties of two extracts, chloroform (CE) and methanolic (ME) extracts, as well as ethyl acetate fraction (AF), butanolic fraction (AC) and aqueous fraction (AQF) of the methanolic extract of the *Abarema cochliacarpos'* bark against acute gastric ulcer. Second, we have selected the AC fraction for assessing its activity in ulcer healing, the roles of the mucus, and the gastric secretion in gastroprotection.

2 Materials and methods

2.1 Animals

Male Wistar rats (150–250 g) and male Swiss mice (25–35g), both obtained from the breeding of the State University of Campinas (CEMIB/UNICAMP), were used. The animals were fed a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of 12 h dark–12 h light, humidity (60±1.0%) and temperature (21±1%). Moreover, the animals were kept in cages with raised floors of wide mesh to prevent coprophagy. The UNICAMP Institutional Animal Care and Use Committee, following the recommendations of the Canadian Council on Animal Care (Olfert et al., 1993), approved all of the employed protocols.

2.2 Vegetal material and extracts preparation

Abarema cochliacarpos (Gomes) Barneby & Grimes was collected (July 2006), in Colônia Treze village, Lagarto city, Sergipe state, Northeastern Brazil by Maria Silene da Silva, and the folk medicine practitioner. Botanical identification was accomplished by Prof. Dra. Crhistine Niezgoda do Field Museum of Natural History. A voucher was deposited under number 007628 at the Federal University of Sergipe. For biological assays, fresh bark of *A. cochliacarpos* was dried 10-14 days. Then it was powdered (1,200g) and macerated with chloroform (3L, 6 days). The solution was filtered through filter paper and the solvents were permitted to evaporate at reduced pressure affording the chloroform extract (CE) with 0.40% yield. After drying, the bark were extracted exhaustively with methanol (Merck, Brazil) successively at room temperature (500 g of material to 2,5 L) 2 weeks. Solvent was evaporated at 40 °C under reduced pressure to yield 34,61% methanolic extract (ME).

The methanolic extract (200.0 g) was partitioned three times with a mixture of ethyl acetate: water (1:1, v/v). The ethyl acetate fraction was evaporated at 35°C under reduced pressure affording an ethyl acetate fraction (AF) of 50.12 g. The aqueous phase was partitioned with a mixture of *n*-butanol: water (1:1 v/v, 3 times), affording 99.69 g of butanolic fraction (AC) and 50.19 g of aqueous fraction (AQF).

2.3 Phytochemical screening

The content of butanolic fraction of *A. cochliacarpos* was investigated by electrospray ionisation (ESI) MS and ESI/MS/MS. Samples were dissolved in MeOH/H2O (1:1 v/v) to obtain a concentration of 0.1%. The solution was subjected to analysis by the ESI ionization source, in the negative ion mode. Scan range was *m/z 50 - 1000* in MS1 mode, 60-340 and 50-1000 MS/MS mode. The nuclear magnetic resonance (NMR) spectra in dimethyl-d6 sulfoxide (DMSO-d6) were obtained using a Varian INOVA 500 spectrometer, operating at 125MHz for 13C. Chemical shifts are given as δ (ppm) using TMS as an internal standard.

2.4 Acute toxicity

The acute toxicity studies were performed in male and female Swiss mice (n = 10). A single dose of the fraction was administered orally to groups of animals after a 12-h fast. Animals receiving 8% Tween 80® served as control. The signs and symptoms associated with the AC administration (5 g/kg, p.o.) were observed at 0, 30, 60, 120, 180 and 240 min after and then once a day for the next 14 days. At the end of the period the number of survivors was recorded and the acute toxicological effect was estimated by the method described by Souza Brito (1994).

2.5 Absolute alcohol-induced ulcer

The animals were divided into five groups of 6 or 7 animals, and were fasted 24 h prior to receiving an oral dose of the vehicle, saline or 8% Tween 80 (10 mL/kg), lansoprazole (30 mg/kg), CE and ME (100, 200 and 400 mg.kg). AF (12.50, 25, 50, 100, 150 and 200 mg/kg), AC (12.5, 25, 50, 100, 150 and 200 mg/kg) AQF (50, 100,150 and 200 mg/kg). After 60 min, all groups were orally treated with 1 mL of absolute ethanol for gastric ulcer induction. One hour later, the animals were sacrificed and their stomachs excised and gastric contents were aspirated. Each stomach was incised along the greater curvature and examined for linear haemorrhagic lesions in the glandular region. Then the stomachs were photographed and the extent of the lesions was measured (mm^2) by AVSoft BioView program (Khan, 2004).

2.6 Determination of mucus in gastric content

Male Wistar rats were divided into groups (n = 7). After animals fasted for 24 h, butanolic fraction of *A. cochliacarpos* (150 mg/kg), Carbenoxolone (200 mg/kg) or the vehicle, 8%Tween 80, was administered orally. Sixty minutes later, under anesthesia, the abdomen was incised and the pylorus ligated. The animals were killed 4 h after the drug treatments. The stomach content was immersed in 10mL of 0.02% Alcian blue 0.16M sucrose/0.05M sodium acetate solution, pH 5.8, and incubated for 24 h at 20°C. The Alcian blue binding extract was centrifuged at 2000×g for 10min. The absorbency of supernatant was measured by spectrophotometry at 598 nm. The free mucus in the gastric content was calculated from the amount of Alcian blue binding [mg/wt tissue (g)]. This assay was performed according to the methodology described by Corne et al, 1974.

2.7 Determination of gastric secretion

The determination of gastric secretion was performed using the method of Shay et al., (1945), with few modifications. The rats were divided into groups (n = 6). After 24 h of fasting, the animals were anesthetized, the abdomen was incised and the pylorus ligated. Immediately after the pylorus ligature, AC was administered by intraduodenal via at doses of 150 mg/kg. Lansoprazole (30 mg/kg) was used as positive control, and 1mL of vehicle (8% Tween-80 aqueous solution) was administered as negative control. All the samples were administered intraduodenally. Four hours later, the animals were sacrificed by cervical dislocation; the abdomen was opened, and another ligature placed around the esophagus close to the diaphragm. The stomachs were removed and the gastric content collected and its contents drained into a graduated centrifuge tube and centrifuged at 2000×*g* for 15min.The supernatant volume and pH were recorded with a digital pH meter (PA 200, Marconi S.A., Brazil).

2.8 Healing action

2.8.1 Acetic acid-induced gastric ulcers

Male Wistar rats (n =5) after having fasted for 24 were used in this experiment. Under anesthesia, laparotomy was performed on all animals through a midline epigastric incision. After exposing the stomach, 0.05 mL (v/v) of a 30% acetic acid solution was injected into the subserosal layer in the glandular part of the anterior wall. The stomach was bathed with saline to avoid adherence to the external surface of the ulcerated region. The abdomen was then closed and the animals were fed normally.

The rats were divided into groups: 8% Tween 80, (10 mL/kg, negative control), lansoprazole (30 mg/kg, positive control) and butanolic fraction of methanolic extract *Abarema cochliacarpos* (150 mg/kg). The animals received the treatments by gavage once a day for 14 consecutive days, beginning 2 days after surgery. During these periods, body weight was recorded daily to evaluate possible chronic toxicity induced by AC. On the day after the last drug administration, the rats were killed and the stomachs were removed. The gastric lesions were evaluated by examining the inner gastric surface with a dissecting magnifying glass. Then the stomachs were photographed and he extent of the lesions was measured (mm^2) by AVSoft BioView program (Khan, 2004). The lesion was sectioned, and fixed in ALFAC solution (alcohol, acetic acid

and formaldehyde) for 24 h at 4 ◦C. Then the samples were routinely processed for embedding in paraplast, cut into 10µm-thick sections and put onto histological slides.

2.8.2 Toxicity evaluation

The toxicological parameters were set according to the method of Souza-Brito (1994). We evalueted the toxicity in the animals submitted to AC treatment under the cicatrisation model described above. For a period of 14 days, AC effects were observed daily (body weight progression, hair and mucosal alteration). The following organs were weighed to detect any effect of the extract on their individual weights: heart, lungs, liver, kidneys and spleen.

2.8.3 Histological analyses

The slides were observed after haematoxylin and eosin (HE) staining (Behmer et al., 1976). The slides had also Periodic Acid Schiff (PAS) staining (Vacca, 1985), in which we observed mucus production. Histological analyses were made using a Leica microscope associated with Leica Q-Win Software 3.1 (Leica-England), from the image analysis laboratory of the Department of Morphology, UNESP-Botucatu.

2.8.4 Immunohistochemical localisation of PCNA, COX-2, vase marker, HSP-70 and VIP

Representative slides of gastric tissue were deparaffinized, rehydrated and immunostained by the peroxidase anti-peroxidase method. High temperature antigen unmasking technique was employed for in 0.01M citrate buffer pH 6.0 in microwave oven, twice for 5 min each, except for HSP-70. Blocking of nonspecific reaction was performed with 3% non-fat milk, and sections were incubated with primary antibodies for PCNA mouse monoclonal antibody (Novo Castra NCL-PCNA) (1:100), COX-2, Cayman Chemical) (1:200), vase marker (VEGF, Novo Castra) (1:50), HSP-70 (Santa Cruz Biotechnology SC-1060) (1:100) and VIP (Novo Castra) (1:100). After rinsing in phosphate buffered saline (0.01 mol/l PBS, pH 7.4), the sections were incubated in secondary antiserum. They were then washed in PBS and incubated in ABC (avidine and biotine complex-Easy Path) and incubated in peroxidase reaction (3,3-diaminobenzidine tetrahydrochloride (Sigma) containing 0.01% H_2O_2 in PBS buffer.

2.9 Statistical analysis

Results were expressed as mean±standard error of means (S.E.M.). The statistical significance of each test group in relation to the control was calculated using ANOVA followed by Tukey's test.

3 Results and Discussion

The genesis of ethanol-induced gastric lesions has a multifactorial origin that induces oxidative stress, DNA damage, and decreases total glutathione content in gastric mucosal cells as some of the involved factors (Sun et al, 1991). Intragastric instillation of absolute ethanol was utilized in control rats and produced large hemorrhagic injury in the glandular stomach. On the other hand, extracts and fractions of *A. cochliacarpos* were able to change this. *Abarema cochliacarpos* CE treatment shows significant reduction in the damaged area ($p<0.01$) at doses 200 and 400 mg/kg when compared with the control group. However, after the ME treatment at 100, 200 and 400 mg/kg oral doses, the data clearly indicated a dose-dependent reduction of the 91.69%, 96% and 99.80% respectively in damaged area. This ME treatment was more inhibitory than the lansoprazole (81.36%) (Table 1). Studies have shown lansoprazole to be a potent dose-dependent inhibitor of acid secretion. It has also been shown that lansoprazole protects against ethanol-induced ulcer formation (Chandranath, Bastaki & Singh, 2002).

These data suggest that ME displays a gastroprotective effect since it significantly reduced ethanol-induced ulcers; thus this extract was chosen for further studies. In order to do this, the partition of this extract was performed and the ethyl acetate fraction (AF), butanolic fraction (AC) and aqueous fraction (AQF), were produced, as explained in item 2.2. AF fraction was not able in reduced gastric lesions by absolute ethanol instillation in the evaluated doses. However, both AC and AQ fractions significantly reduced these lesions, but AC was more effective in the low dose (150 mg/kg) when compared with AQ fraction (200 mg/kg) (table 2). Moreover, AC fraction is less polar than AQ fraction, what facilitates the isolation of compounds. The phytochemical screening of the AC fraction performed by our group showed high content polyphenols (like tannins) and (+)-catechin is the major constituent. Considering these points, and the strong association of these compounds with anti-inflammatory and anti-ulcerogenic effects and healing, the butanolic fraction

of methanolic extracts of *Abarema cochliacarpos* was chosen for further studies to clarify the mechanisms underlying its gastroprotective activity.

AC was first investigated for acute toxicity in mice. A single oral dose of AC (5 g/kg) did not produce any visible signs or symptoms of toxicity in the treated animals. After 14 days of administration, no animal died, and no significant macroscopic changes in daily body or organ weights were observed (data not shown). Since no acute toxicity was observed using AC, we continued our studies evaluating the mechanisms underlying the gastroprotective activity of *Abarema cochliacarpos*.

As shown in Table 3, the pretreatment with AC (150 mg/kg) induced a significant increase in the mucoprotective effect in animals submitted to pylorus ligation, with the main free mucus being significantly increased as compared to the mucus barrier of control animals ($p<0.05$), definitely indicating an increase of mucus production by the gastric mucosa (47.8%), which strongly suggested the involvement of a process of mucosal adaptation by means of an increase in the activity of defense mechanisms. These results are in accordance with Hamaishi et al. (2006) showed who that tea catechin oral doses administration increased the gastric hexosamine content in rats, turn Faria (2009) by his turn demonstrated that phenolic compounds of butanolic fraction of *Rhizophora mangle* increased mucus production in the gastric mucosa in rats subjected to pylorus ligation.

In the pylorus-ligated rat, AC (150 mg/kg) administered intraduodenally presented significant difference between 8% Tween 80 and the AC group in the analyzed parameters (pH and gastric secretion volume). Lansoprazole, at 30 mg/kg, significantly reduced gastric juice volume and gastric juice acidity (Table 4). Thus, our findings suggest that the gastroprotective action of AC is involved in antisecretory activity. Faria (2009) showed similar results with butanolic fraction of *Rhizophora mangle,* wich is also rich tannins. And an interesting study by Rao & Vijayakumar (2007) using the model of ischemia and reperfusion in rats, demonstrated that (+)-catechin was able to decrease the levels of H^+ K^+ATPase as compared with control rats.

Confirmation of an AC gastroprotective effect does not mean that this same extract also resents healing and cicatrisation actions in gastric ulcers still present in the mucosa. The so-called acetic acid ulcer model has been developed to examine the healing process of peptic ulcers.

In this acetic acid model, oral treatment with AC of *A. cochliacarpos* for 14 consecutive days demonstrated that it accelerates the healing of chronic gastric ulcer in rats (Fig. 1). In addition, AC150 significantly decreased the main area of the lesion, 49.81±3.93 mm^2 vs. 103.80±5.73mm^2 in

the negative control ($p<0.001$). The antiulcerogenic drug, lansoprazole, shows significant differences from the group treated with 8% Tween 80 after 14 days ($54.67\pm6.97mm^2$). These data show a strong healing activity of AC150 of *A. cochliacarpos,* which was observed both in the macroscopic and morphological analysis of the injuries. This model highly resembles human ulcers in both pathological features and healing mechanisms since they are difficult to treat and require a long time to heal (Okabe and Amagase, 2005). It also resembles the popular use of this medicinal plant.

Another important finding was that this experimental model can provide the toxicological parameters from animal treated with AC for two consecutive weeks. There was no significant difference in body weight development (Fig. 2) or organ weights (Table 5) for all groups. No macroscopic abnormalities were detected in the examined organs. Nor was mortality observed in any treatment group during the 14 days study.

Ulcer healing, a genetically programmed repair process, includes inflammation, cell proliferation, reepithelialization, formation of granulation tissue, angiogenesis, interactions between various cells and the matrix and tissue remodeling, all resulting in scar formation (Tarnawski, 2005).

In the HE staining significant increase has been observed in the regeneration area of the mucosa during the 14 days of AC treatment, when compared with animals treated with vehicle (Fig.3). The morphological analysis showed a difference in the organization of the tubular mucosa in the AC group. However, glands apparently showed appearance similar to those treated with lansoprazole and mucus secretion, according with the results by PAS staining (Fig. 4). In the 14-day AC treatment (Fig. 4E and F), glands showed an increased mucus production when compared to histological analysis from the gastric mucosa of the tween group (Fig. 4A and B).

The histological analysis was in accordance with the proliferating cell nuclear antigen (PCNA), a cell proliferating marker. This is an immunohistochemical analysis that reveals a great number of proliferating cells in the stomachs treated with AC (Fig.5C) for 14 days as compared with the negative control (Fig. 5A) or lansoprazole (Fig. 5B). PCNA, a highly conserved 36 kDa nuclear peptide, was identified as the auxiliary protein of DNA polymerase delta (Bravo et al., 1987; Prelich et al., 1987). It is expressed mainly during the S-phase of the cell cycle, and therefore, in proliferating cells, where it functions to initiate and choose different bypassing pathways of post-replication repair (Celis and Madsen, 1986).

The capacity to accelerate the ulcer healing process depends on many factors, like the EGF-epidermal growth factor, bFGF- fibroblast growth factor, VEGF-vascular endothelial growth factor, trefoil peptides and COX-2 in a well synchronized spatial and temporal manner (Tarnawski, 2005).

On what regards the COX-2 and VEGF immunohistochemical analysis, our results showed a great quantity of COX-2 expression in the regenerative region of the gastric mucosa of animals treated with AC150 (Fig. 6C) as compared to the vehicle-treated group (Fig. 6A). There was an increase of the vascular-endothelial growth factor (VEGF) in granulation tissue, as ulcer margins (Fig. 7C). Moreover, a great number of newly formed vessels were noticeable in the AC after 14 days treatment (Fig. 7C).

In fact, COX-2 expression hadn't been shown in the gastric mucosa until Mizuno et al., 1997 described that COX-2 mRNA and the protein were induced in gastric mucosa bearing erosion and ulceration. COX-2 plays an important role in the healing of gastric ulcers whereas COX-2 inhibition delays ulcer healing (Peskar, 2005). The direct involvement of COX-2 in ulcer healing has been supported by observations of upregulated expression of COX-2 mRNA at the ulcer margin both temporally and spatially in relation to enhanced epithelial cell proliferation and increased expression of growth factors (Halter et al., 2001).

VEGF functions as an endothelial cell mitogen, chemotactic agent, inducer of vascular permeability, and it is unique for its effects on multiple components of the wound-healing cascade, including angiogenesis and recently shown epithelialization and collagen deposition (Stojadinovic et al, 2007; Bao et al., 2009). *In vitro* studies performed by Miura et al. (2004) in human gastric fibroblasts, suggested that the VEGF production by PGE_2-induced in COX-2-expressing fibroblasts might play a key role in angiogenesis. These authors also show that the double staining of COX-2 and VEGF with immunofluorescence-conjugating antibodies revealed that VEGF and COX-2 were co-expressed in these spindle-shaped and spherical cells of the ulcer bed or gastric human tissue. In agreement with this data, our results demonstrated an augment of immunolocalization of both COX-2 and VEGF in the gastric mucosal tissue, strongly suggesting that these substances participate in the mechanism of action underlying the activity of healing ulcer de *Abarema cochliacarpos*.

Another important observation of the present study was an increase in the expression of heat shock protein 70 (HSP70) in the ulcerated mucosa in the animal group treated with AC150 mg/kg of *A. cochliacarpos* (Fig. 8C). HSP-70 is an important member of the heat shock protein family also known as stress protein, and it is involved in different essential cellular events such as

folding, assembling, and transport of proteins (Feder et al., 1999). This finding is keeping with previous studies showing an increase in HSP70 expression after the damage of the gastric mucosa (Schichijo et al., 2003; Yanaka et al., 2007). In the case of wound healing, HSP70 was expressed in proliferating cells during reepithelization (Soncin & Calderwood, 1996) thus it is thought that the HSP70 expression in the ulcer margin is also linked to the regeneration of the ulcerated mucosa. HSP70 induced in the ulcer base might either contribute to *de novo* synthesis of proteins or regulate the activity of key enzymes for ulcer healing, like COX-2 and growth factors, through molecular chaperone activity (Tsukimi & Okabe, 2001).

The VIP expression was also investigated. The results demonstrate that the group treated with antiulcerogenic drug, lansoprazole, did not show expression of this protein. However, its expression occurred in the gastric submucosal tissue of the AC150-treated group, but it was strongly markedly in the vehicle.treated group (data not shown).

Thus, we demonstrated that CE and ME extracts of *Abarema cochliacarpos* have gastroprotective activity in the ethanol induced ulcers model, and the butanolic fraction of methanolic extracts of *Abarema cochlia*carpos protects gastric mucosa from acute gastric mucosal injury and promotes the healing of chronic ulcers probably by its gastric mucus-increasing and augment of PCNA, COX-2, VEGF and HSP-70 expressions in the gastric tissue.

4 Conclusion

All these results suggest that *Abarema cochliacarpos* (Gomes) Barneby & Grimes presents gastroprotective effects and wound healing properties. The safety and efficacy in the healing of gastric ulcers is based on its ability to activate the expression of COX-2 and VEGF and stimulate proliferative factors that re-establish the gastric mucosa integrity.

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Figure 1

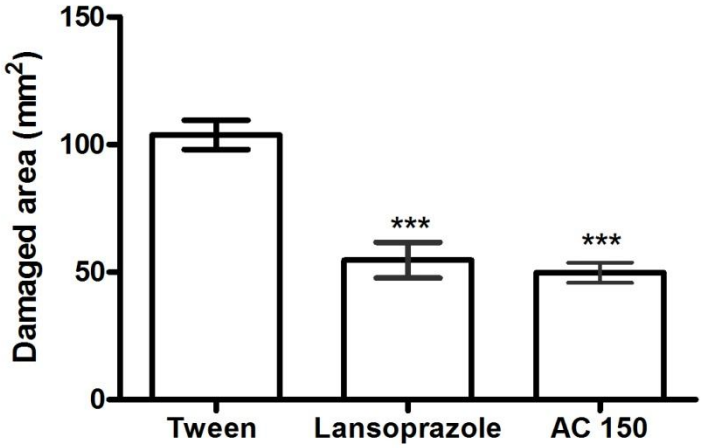

Figure 1- Effects of chronic administration of AC 150 mg/kg of *Abarema cochliacarpos* on the healing ulcerin rats with chronic ulcer induced by 0.05 ml (v/v) of a 30% acetic acid solution.This doses significantly decreased the extent and severity of damaged area by acetic acid at 14 days. Data are expressed as the means ± S.E.M. One way ANOVA. Tukey's test: (***) $p<0.001$ vs. vehicle group treated.

Figure 2

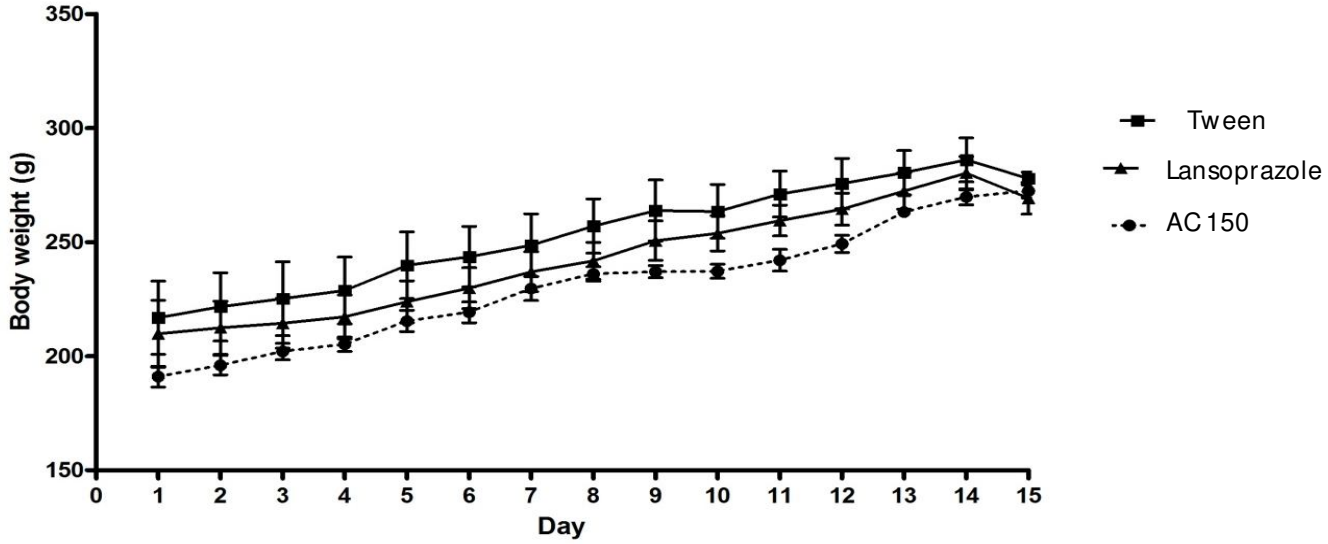

Figure 2 -Body weight gain in rats treated orally with vehicle, lansoprazole (30 mg/kg) or butanolic fraction of metanolic extract from *Abarema cochliacarpos* (AC150 mg/kg) for 14 days after ulcer formation by acetic acid solution injected into the stomach.

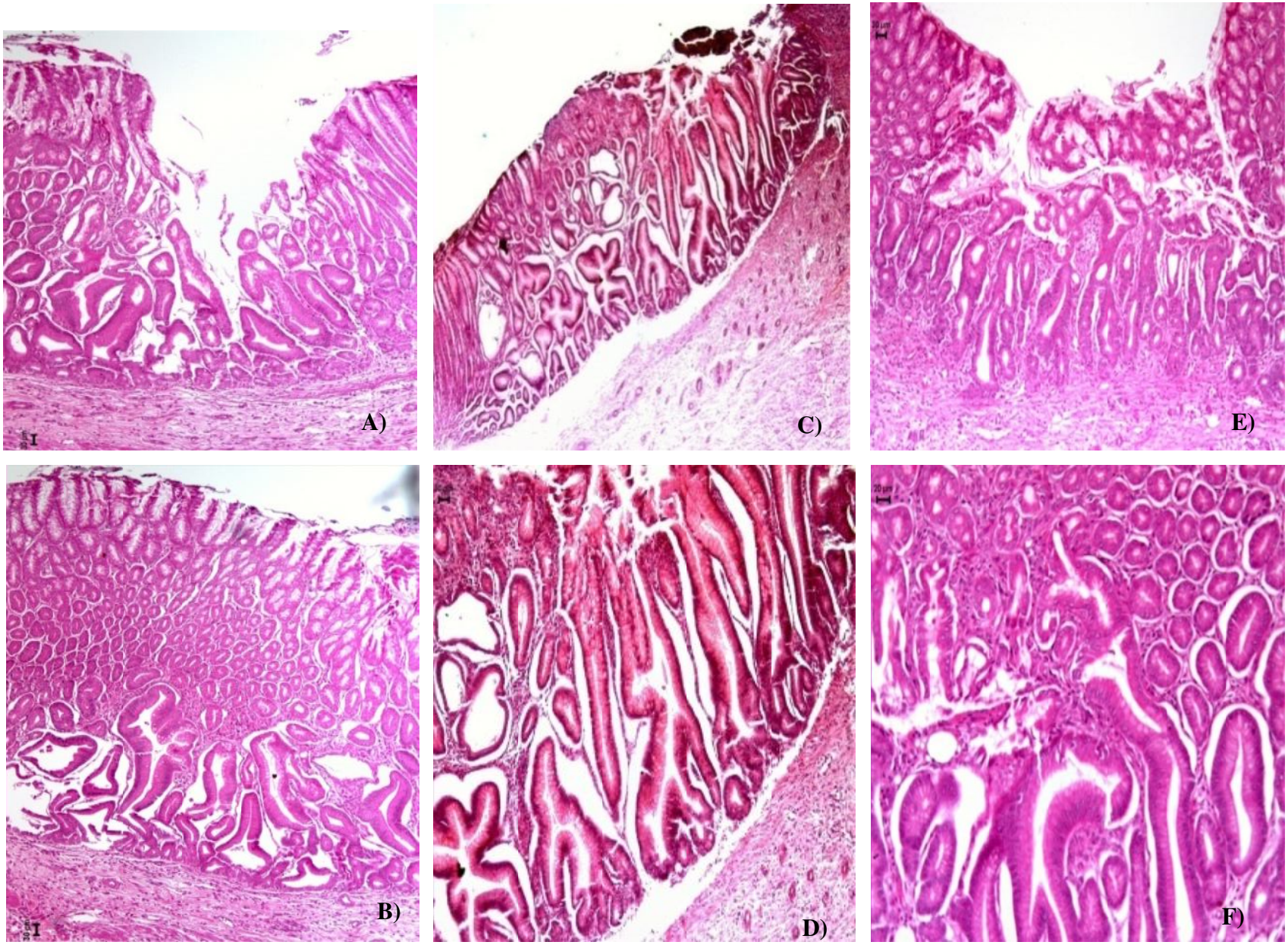

Figure 3 - Histological analysis of rats' stomachs treated with 8% Tween (10 ml/kg) (A and B), Lansoprazole (30 mg/kg) (C and D), and AC *Abarema cochliacarpos* (150 mg/kg) (E, and F), for 14 days, in haemtoxylin and eosin staining. Notice in AC (E) regeneratrion area lesion, and (F) and dilated glands with much secretion. Original magnifications. A, C and E – 20x; B, D and F – 40x.

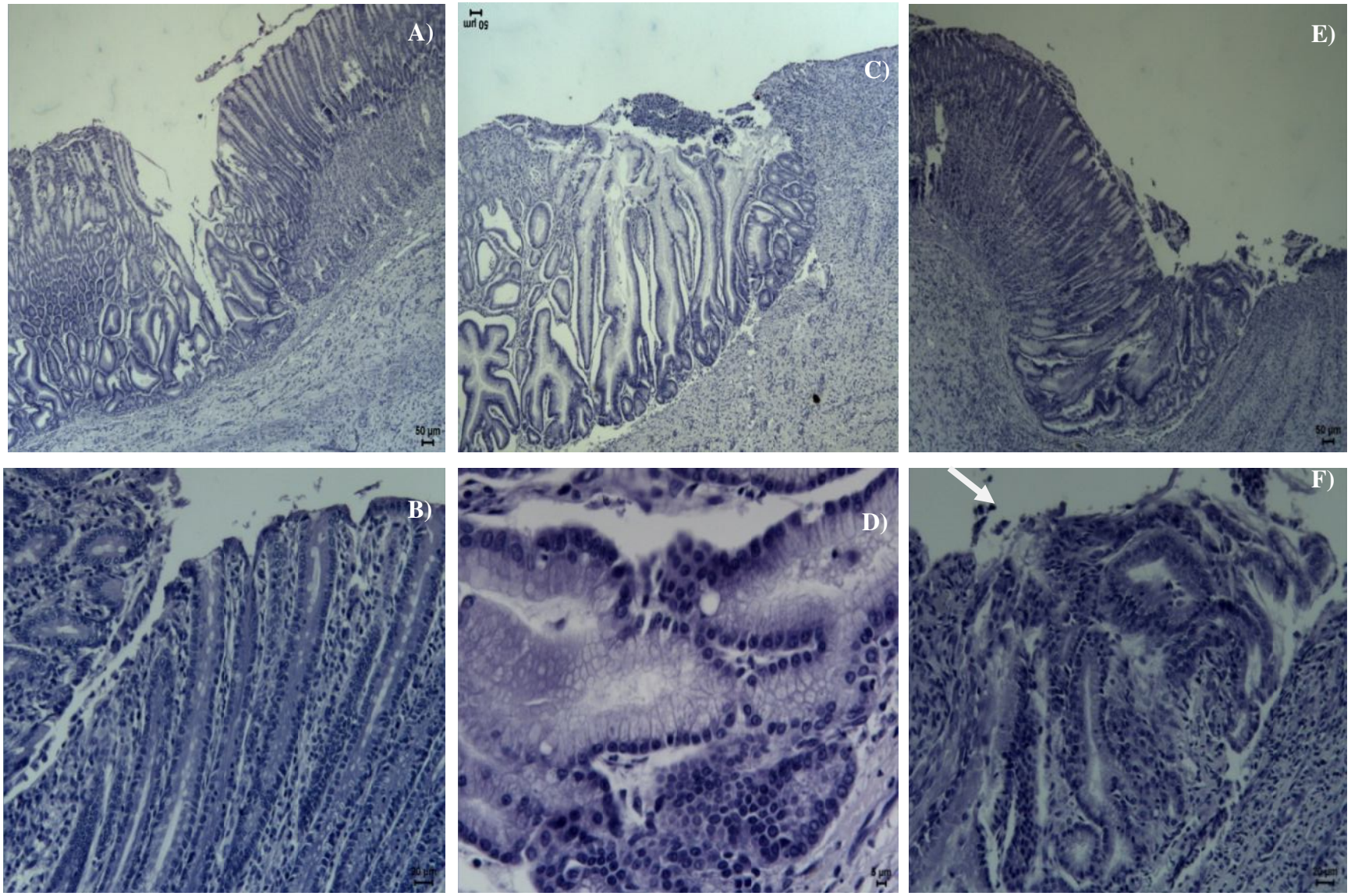

Figure 4 - Histological analysis of rats stomachs treated with 8% Tween 80 (10 mL/kg) (A and B), lanzoprazole (30 mg/kg) (C and D) and AC (*Abarema cochliacarpos*-150 mg/kg), for 14 days (E-F), in PAS staining. Notice the great amount of mucus secretion in AC (arrows). Original magnifications. A and C – 20x; B – 10x.

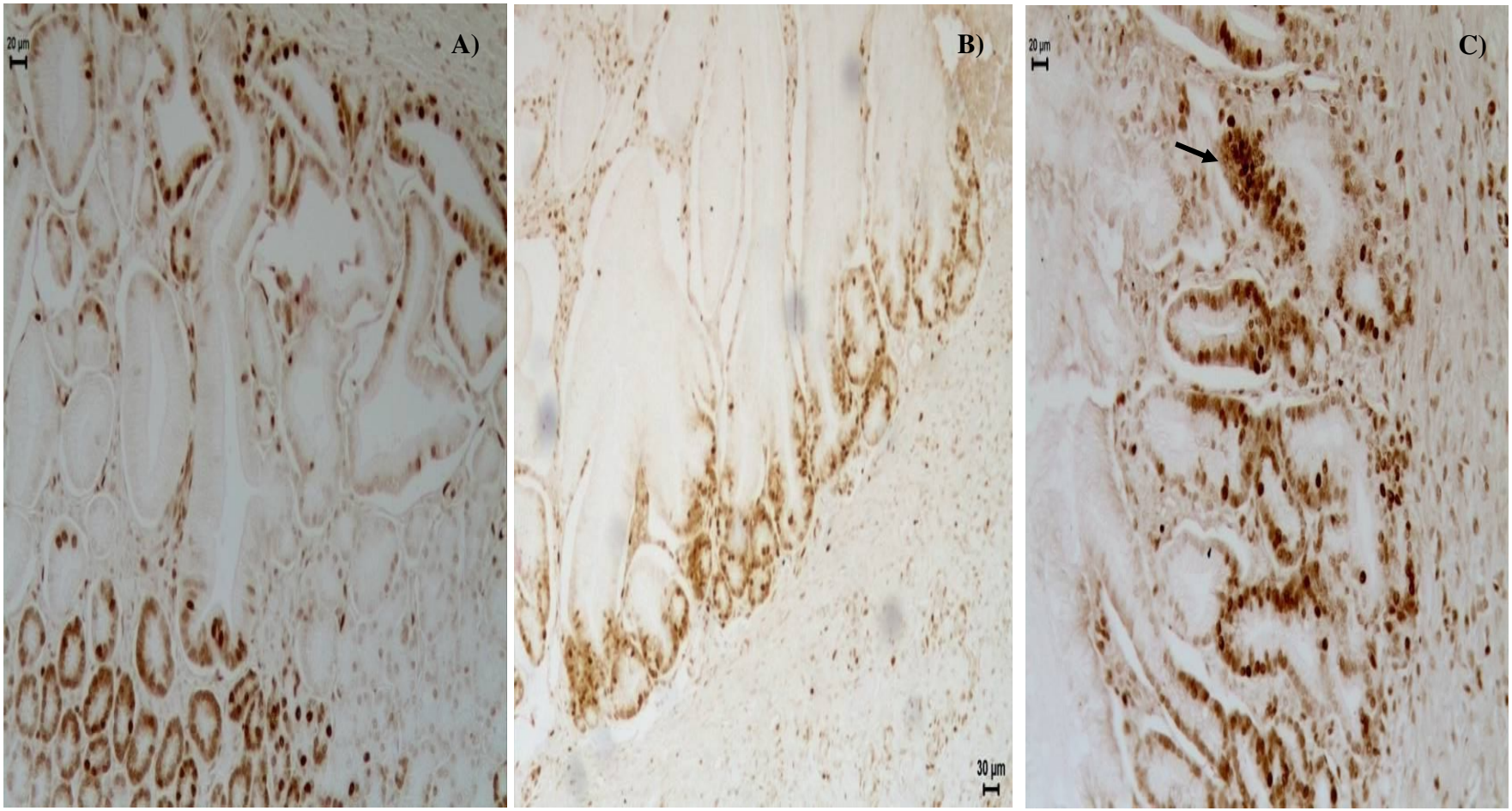

Figure 5 -Histological analysis of rats' stomachs treated with 8% Tween 80, Lansoprazole (30 mg/kg) and AC (*Abarema cochliacarpos* 150 mg/kg) for 14 days (A–C) respectively in PCNA immunohistochemistry. Notice the great number of PCNA-positive nuclei in AC (dark spots indicated by arrows). Original magnifications. A and C – 20x; B – 10x.

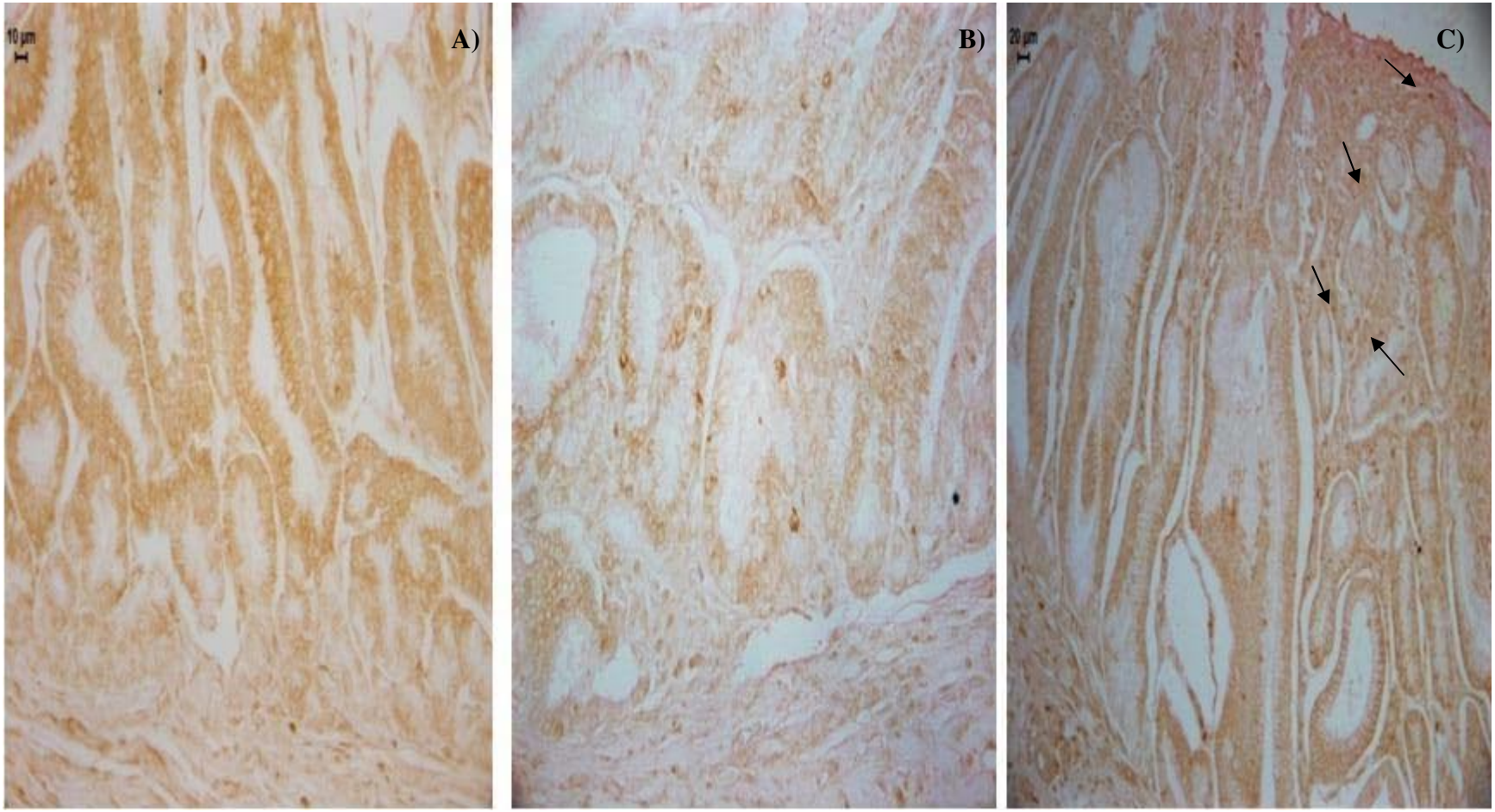

Figure 6 - Histological analysis of gastric mucosa of rats treated with 8% Tween 80 (A), Lansoprazole (B), and butanolic fraction (AC) (C) from *Abarema cochliacarpos* (150 mg/kg) for 14 days by COX-2 immunohistochemistry. Note the great quantity of COX-2 staining indicated by arrows. Original magnifications: A and B – 40x; C – 20x.

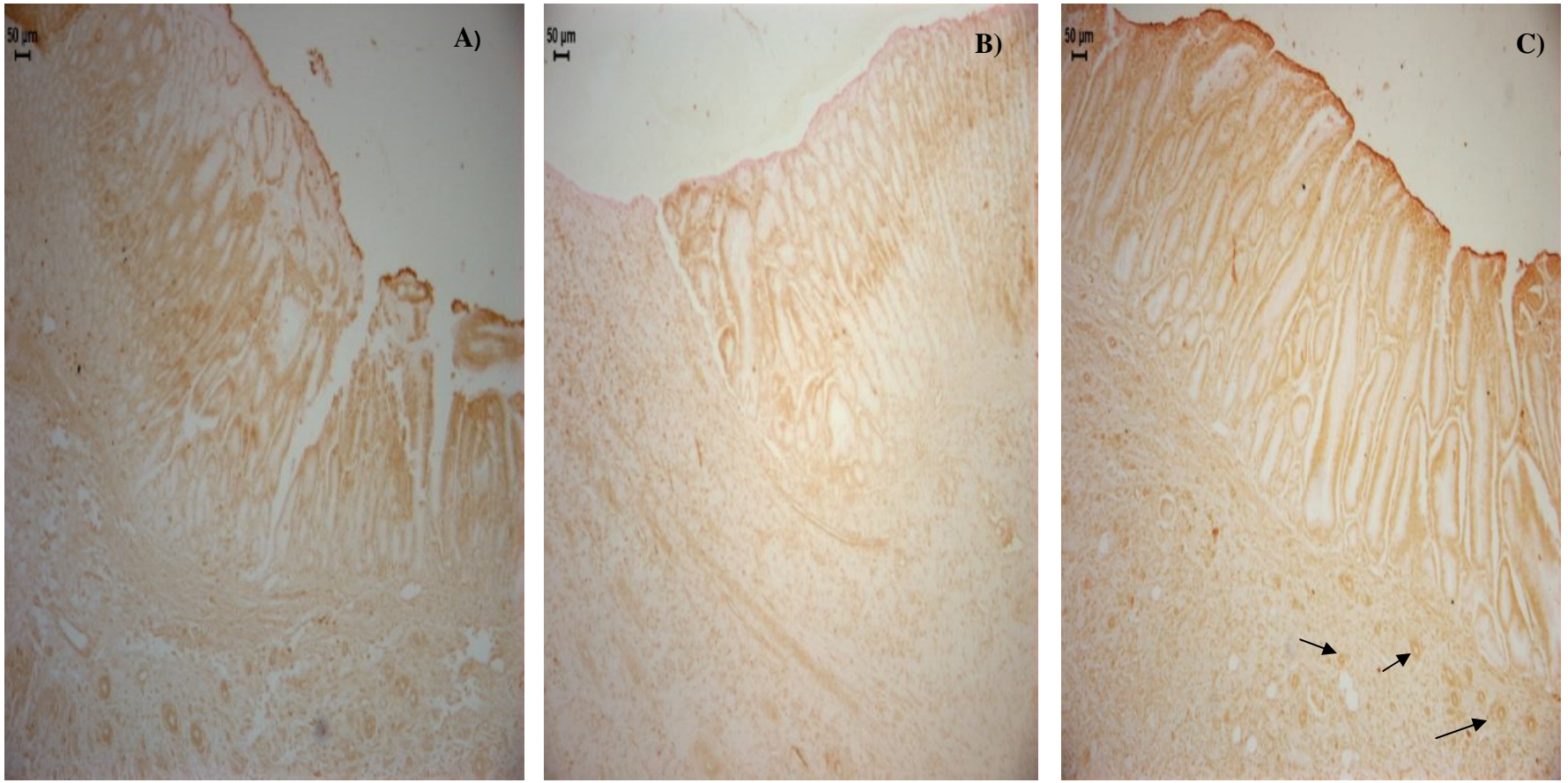

Figure 7 - Histological analysis of gastric mucosa of rats treated with 8% Tween 80 (A), Lansoprazole (B), and butanolic fraction (AC) from *Abarema cochliacarpos* (150 mg/kg) for 14 days by vase marked (VEGF) immunohistochemistry. Note the great quantity of vessels marked in granulation tissue indicated by arrows. Original magnifications: 10x.

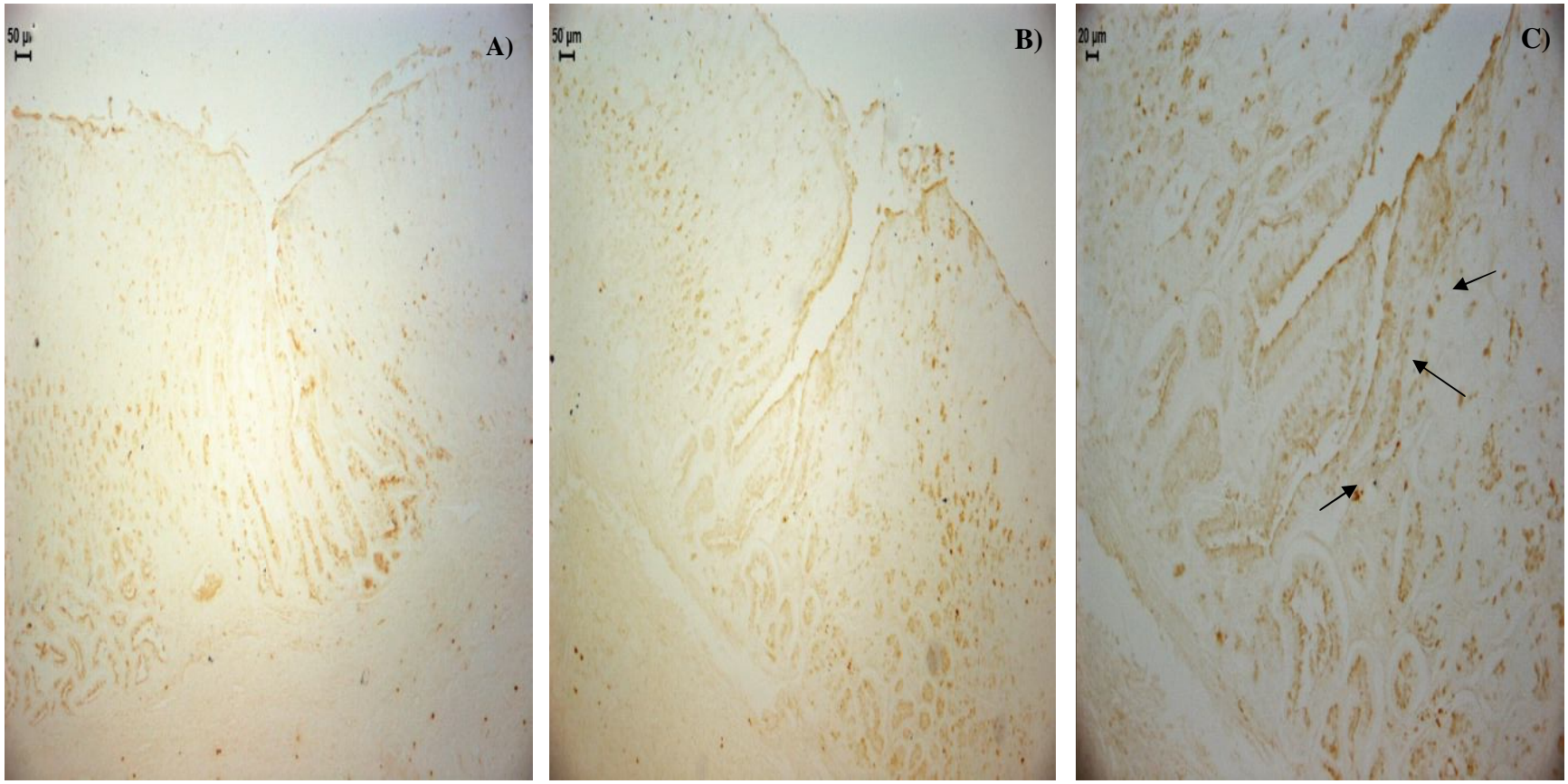

Figure 8 - Histological analysis of gastric mucosa of rats treated with 8% Tween 80 (A), butanolic fraction (AC) from *Abarema cochliacarpos* (B and C), for 14 days by HSP-70 immunohistochemistry. Note the great quantity of HSP-70 staining indicated by arrows. Original magnifications: A and B – 10x; C - 20x.

Table 1

Effects of the *A. cochliacarpos* bark chloroform (CE) and methanol (ME) extracts on ethanol-induced gastric damage in rats.

Treatments (p.o.)	Dose (mg/kg)	N	Damaged area (mm^2)	Inhibition(%)
8% Tween 80	_	8	15.78±1.21	—
Lansoprazole	30	7	4.18±1.05***	69
CE	100	5	13.98±3.55	26
	200	5	6.56±1.036**	65
	400	5	3.06±0.26**	83
Saline	_	7	76.62±18.60	—
Lansoprazole	30	7	14.29±5.65**	81
ME	100	5	8.90±4.68**	92
	200	5	3.47±2.13***	97
	400	5	0.21±0.21***	100

Results are mean±S.E.M. CE-ANOVA one way F(4,23) = 2.64 (P<0.001); ME -ANOVA one way F(4,24)=2.62 Tukey's test (P<0.01); **P<0.01 and ***P<0.001 versus negative control.

Table 2

Effects of the partitioned of *A. cochliacarpos* bark methanolic extracts (ME) on ethanol-induced gastric damage in rats.

Treatments (p.o.)	Dose (mg/kg)	N	Damaged area (mm^2)	Inhibition(%)
8% Tween 80	_	7	72.50±11.30	___
Lansoprazole	30	7	14.49±3.48**	80
AF	12.50	6	29.86±8.41	65
	25	6	34.11±13.14	60
	50	6	31.96±4.84	62
	100	7	46.29±11.52	36
	150	7	32.96±8.19	55
	200	6	55.18±15.26	35
8% Tween 80	_	7	43.83±6.40	__
Lansoprazole	30	7	9.56±4.38**	79
AC	12.50	6	48.31±10.14	___
	25	6	27.75±5.54	30
	50	6	38.78±6.64	12
	100	6	20.62±2.05	53
	150	6	9.67±3.56**	74
	200	6	3.91±1.88***	90

Table 2 continued

Saline	_	7	80.13±14.43	__
Lansoprazole	30	6	5.72±1.37**	92
AQF	50	5	37.70±10.68	61
	100	6	50.58±19.46	37
	150	6	30.45±13.93	62
	200	6	12.71±18.81**	84

Results are mean±S.E.M. AF-ANOVA one way F(7,44) = 2.25 (P<0.01); AC-ANOVA one way F(7,42) = 2.25 (P<0.01); AQ-ANOVA one way F(5,30) = 2.53 (P<0.001). Tukey's test,**P<0.01; Tukey's test ***p<0.001 vs negative control.

Table 3

Effects of butanolic fraction of *Abarema cochliacarpos* bark administered orally on Alcian blue binding to free gastric mucous from pylorus ligature rats

Treatments	**Dose (mg/kg)**	**N**	**Alcian blue bound (mg/wt tissue (g))**
8% Tween 80	__	7	28.94 ± 1.40
Carbenoxolone	200	7	46.59 ± 4.04*
AC	150	8	42.57±3.54*

Results are mean±S.E.M. ANOVA one way F(2,18) =3,55; Tukey's test: *p < 0.05.

Table 4

Effects of butanolic fraction of methanolic extract of *A. cochliacarpos* by intraduodenal route on biochemical parameters of gastric juice obtained from pylorus ligature rats.

Treatments	Dose (mg/Kg)	pH (units)	Volume (mL)
8% Tween 80®	___	1.88±0.29	2.88±0.25
Lansoprazole	30	3.12±0.27**	2.004±0.20*
AC	150	3.08±0.16*	2.05±0.20*

Results are mean±S.E.M. ANOVA one way F(2,17) = 2,17; Tukey's test: *p<0.05, **p<0.01 vs negative control.

Table 5

Relation organs weight/body weight of rats after oral treatment with 8% Tween, Lansoprazole (30 mg/kg) or *A. cochliacarpos* (AC, 150 mg/kg) for 14 consecutive days.

Treatment	Heart	Lung	Kidneys	Liver	Testicle
Tween	3.48±0.06	4.42±0.13	5.00±0.09	10.88±0.32	6.17±0.17
Lansoprazole	3.46±0.09	4.21±0.10	5.19±0.15	11.68±0.40	6.018±0.08
AC	3.46±0.02	4.58±0.24	5.20±0.06	11.44±0.13	5.95±0,13

The values were transformed into arc sine. Results are mean±S.E.M. ANOVA one way, p > 0.05. 14 days (*n* = 5,5,5) respectively, for Tween, lansoprazole and AC.

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Anti-inflammatory intestinal activity of *Abarema cochliacarpos* (Gomes) Barneby & Grimes in TNBS colitis model

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ABSTRACT

Aim of the study: To assess the anti-inflammatory effect of butanolic fraction of methanolic extract from bark of *Abarema cochliacarpos* in acute ulcerative colitis model induced by intracolonic administration of trinitrobenzene sulfonic acid (TNBS) in Wistar rats.
Materials and methods: *Abarema cochliacarpos* (100 and 150 mg/kg/day) was administered by gavage 48, 24 and 1 h prior to the induction of colitis with 10 mg/kg of TNBS and, 24 h later.
Results: Phytochemical studies by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) revealed that catechins were a major component into condensate class of tannins. Treatment with *Abarema cochliacarpos* decreased significantly macroscopic damage as compared with TNBS ($p < 0.05$). Histological analysis showed that both doses of the extract improved the microscopic structure and preserved some areas of the colonic mucosa structure. In addition, myeloperoxidase activity (MPO), as a marker of neutrophil infiltration, was decreased in a dose-dependent way ($p < 0.01$ and $p < 0.001$ respectively), TNF-α level was also diminished with the highest dose of the extract ($p < 0.001$) and, IL-10 level obtained no significant results. In order to elucidate some of the mechanisms, expression of inducible inflammatory enzymes, such as cyclooxygenase (COX)-2 and nitric oxide synthase (iNOS), were studied showing a significant reduction. Finally, the involvement of c-Jun N-terminal kinase (JNK) signalling demonstrated a reduction in the JNK activation with the highest dose ($p < 0.05$ vs TNBS).
Conclusions: We have shown for the first time that the extracts obtained from *Abarema cochliacarpos* bark possess active substances, which exert marked protective effects in acute experimental colitis, confirming and justifying, at least in part, the popular use of this plant to treat gastrointestinal diseases.

1. Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease, is a chronic intestinal disorder resultant from a dysfunctional epithelial, innate and adaptive immune response to intestinal microorganisms. Its clinical course is unpredictable and presents remissions and exacerbation, characterized by rectal bleeding and diarrhoea leading to disruption of the epithelial barrier, and the formation of epithelial ulceration. In addition, the development of an abnormal immune and inflammatory response occurs, which is mediated predominantly by activated neutrophils, monocytes and macrophages and characterized by an enhanced formation of reactive oxygen and nitrogen species (Martin et al., 2006). Furthermore, these can activate diverse signalling pathways which lead to the activation of transcription factors such as nuclear factor kappa β (NF-κβ) or activator protein-1 (AP-1), modulating a number of different steps in the inflammatory cascade. These include production of pro-inflammatory cytokines as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, interferon (INF)-γ, IL-12, and IL-6 in

Abbreviations: ANOVA, one-way analysis of variance; AP-1, activator protein-1; COX-2, cyclooxygenase -2; EDTA, ethylenediaminetetraacetic acid; HETAB, hexadecyl-trimethylammonium bromide; IBD, inflammatory bowel disease; IFN-γ, interferon γ; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MPO, myeloperoxidase; NF-kβ, nuclear factor kappa β; NMR, nuclear magnetic resonance spectroscopy; PBS, potassium phosphate buffer; TNBS, trinitrobenzene sulfonic acid; TNF-α, tumour necrosis factor alpha.

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different cell-types, degranulation of neutrophils, as well as the expression of important determining parameters meanly cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) (Collino et al., 2006; Pecchi et al., 2009). Therefore, imbalance between pro-inflammatory and anti-inflammatory cytokines and inflammatory proteins expression including COX-2 and iNOS, which are expressed as an early response to pro-inflammatory mediators and mitogen stimuli, plays an important role in the pathophysiology of this disease (Talero et al., 2008). Moreover, recent studies have reported that activation of mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK) plays an important role in the intestinal inflammation in patients with IBD (Roy et al., 2008).

Abarema cochliacarpos (Gomes) Barneby & Grimes, popularly known as "barbatimão", is a Brazilian native plant occurring mainly in the Atlantic Forest and in the caatinga biomes. It is a tree species in the legume family Mimosaceae (IUCN, 2009). Many traditional communities of Northeastern Brazil utilize bark of *Abarema cochliacarpos* in popular medicine. Decoction of steam bark is used for wash external ulcers and, and the ingestion of this decoction or the tincture, which is made by placing the bark in white wine or "cachaça" is used against inflammation and gastric ulcers (Silva, 2003; Silva et al., 2006). Others authors also observed similar use in different traditional communities (Agra et al., 2008). Study from Santos (2008) shows the versatile uses for tea from the bark in inflammation, pain, cancer and abdominal pain of cause not determinate. Silva (2006) also indicates a popular use like analgesic.

The antiulcer effect of the aqueous extract of stem bark of *Abarema cochliacarpos* (Gomes) Barneby & Grimes was tested in experimental alcohol gastric ulcers demonstrating for the first time a reduction of the lesions (Silva et al., 2006). The capacity of bacterial growth inhibition *in vitro* for the extract hydroalcoholic of the bark has been confirmed by some authors (Araújo et al., 2002; Santos et al., 2007) and more recently, a crude aqueous and methanol extracts also showed antinociceptive effects (Silva et al., 2009), corroborating its popular stories.

Catechins, such as catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate, are particularly abundant in steam bark of the species of Mimosaceae family (Santos et al., 2002). Primary phytochemicals analysis was performed by the phytochemical group and was revealed that crude and methanolic extracts of *Abarema cochliacarpos* gave positive results for saponins, catechins, tannins, phenols and anthraquinones (Silva et al., 2009).

Catechins and proanthocyanidins, belonging to the flavonoids, have been evaluated in preclinical IBD models demonstrating promising results (Mazzon et al., 2005; Dryden and Song, 2006). The mechanisms by which catechins achieve their beneficial effects are still not entirely clear; however, there is mounting evidence that they likely work through a combination of both antioxidant effect and alteration of intracellular signalling.

Starting from these dates and the previous studies of our group, which have recently observed that polyphenols have a significant preventive effect on both acute and chronic phases of TNBS-induced colitis in rats (Martin et al., 2006; Camacho-Barquero et al., 2007), we considered of interest to study the pharmacological activity of *Abarema cochliacarpos* in a experimental model of IBD in order to evaluate a novel therapeutic approaches for its treatment.

From these points of view and to prove the popular use of this plant to treat gastrointestinal diseases seem interesting to study the pharmacological activity of steam bark of *Abarema cochliacarpos* as anti-inflammatory in TNBS-induced acute colitis. In this way, we have assessed the inflammatory response by histological analysis as well as by determination of inflammation markers such as MPO activity and cytokines profile. Inducible COX-2 and iNOS expressions and JNK activation changes by Western blotting have also been evaluated.

2. Materials and methods

2.1. Plant material

Abarema cochliacarpos steam bark was obtained from collection by Maria Silene da Silva and the folk medicine practitioner, in Colônia Treze village, Lagarto city, Sergipe state, Brazil, in July 2006. Voucher specimens of this specie were identified by Prof. Dra. Christine Niezgoda from Field Museum of Natural History and were deposited under number 007628 at the Federal University of Sergipe.

2.2. Preparation of the extract

Fresh bark of *Abarema cochliacarpos* was dried at room temperature for 10–14 days. Then it was powdered (1200 g) and exhaustively extracted with methanol (5 l) at room temperature during 15 days by maceration. The suspension was concentrated at 40 °C under reduced pressure to provide a crude methanolic extract with 34.61% yield (415.4 g).

The methanolic extract (200 g) was partitioned three times with a mixture of ethyl acetate:water (1:1, v/v). The ethyl acetate fraction was evaporated at 35 °C under reduced pressure affording an ethyl acetate fraction of 50.12 g. The aqueous phase was partitioned with a mixture of *n*-butanol:water (1:1, v/v, 3 times), affording 99.69 g of butanolic extract fraction and 50.19 g of aqueous fraction.

2.3. Phytochemical screening

The content of butanolic fraction of *Abarema cochliacarpos* was investigated by electrospray ionization (ESI) MS and ESI/MS/MS. Samples were dissolved in MeOH/H_2O (1:1, v/v) to obtain a concentration of 0.1%. The solution was subjected to analysis by the ESI source, in the negative ion mode. Scan range was *m/z* 50–1000 in MS1 mode, 60–340 and 50–1000 MS/MS mode. The nuclear magnetic resonance (NMR) spectra in dimethyl-d6 sulfoxide (DMSO-d6) were obtained using a Varian INOVA 500 spectrometer, operating at 125 MHz for ^{13}C. Chemical shifts are given as δ (ppm) using TMS as an internal standard.

2.4. Animals

Male and female Wistar rats supplied by Animal Services of the University of Seville, Spain, weighing 180–200 g, were placed in singled in cages with wire-net floors in a controlled room (temperature 24–25 °C, humidity 70–75%, lighting regimen of 12L/12D) and were fed a normal laboratory diet (Panlab, Barcelona, Spain). Rats were deprived of food for 12 h prior to the induction of colitis, but were allowed free access to tap water throughout. They were randomly assigned to groups of 8–13 animals. Experiments followed a protocol observed by the Animal Ethics Committee of the University of Seville and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EC).

2.5. Induction of colitis

Colitis was induced according to the procedure described by Morris et al. (1989). Briefly, rats were slightly anesthetized with 12% chloral hydrate by intraperitoneal route following a 12 h fast, and then a medical-grade polyurethane cannula for enteral feeding (external diameter 2 mm) was inserted into the anus and the

tip was advanced to 8 cm proximal to the anus verge. TNBS (Sigma-Chemical Co., St. Louis, MO, USA) dissolved in ethanol (50%, v/v) was instilled into the colon through the cannula (10 mg in a volume of 0.25 ml) to induce acute colitis. A reference control group was applied for comparison with the TNBS colon instillation group: the sham group that received physiological saline instead of the TNBS solution in a comparable volume. Sham and TNBS groups also received the vehicle (2% Tween 80 in a volume of 10 ml/kg body weight) by oral route.

Following the instillation of the hapten, the animals were maintained in a head-down position for a few minutes (2–3 min) to prevent leakage of the intracolonic instillate. Butanolic fraction of *Abarema cochliacarpos* (100 and 150 mg/kg) was suspended in 2% Tween 80 (Sigma-Chemical Co., St. Louis, MO, USA) (Santos et al., 2004) and it was administered in a volume of 10 ml/kg body weight by oral route 48, 24 and 1 h prior to the induction of colitis and also 24 h later. Animals were sacrificed, using an overdose of anesthetic, 48 h after induction of colitis.

2.6. Assessment of colitis

An independent observer who was blinded to the treatment evaluated the severity of colitis. For each animal, the distal 10 cm portion of the colon was removed and cut longitudinally, slightly cleaned in physiological saline to remove fecal residues and weighed. Macroscopic damage was quantified measuring the extent of the lesions in the distal colon (mm^2) using Bioview 4 (AvSoft, Brazil) (Khan, 2004). The presences of adhesions (scores 0–2), and/or stool consistency (score 0–1) were evaluated according to the criteria of Bobin-Dubigeon et al. (2001), with slight modifications. Photographs taken from colon samples were digitized using Kodak D290 Zoom camera (Eastman Kodak Co., Rochester, NY, USA). Pieces of colon were collected and frozen in liquid nitrogen for measurement of biochemical parameters.

2.7. Histological studies

For examination with the light microscope we used tissue samples from the distal inflamed colon of each animal and fixed in 4% buffered formaldehyde, then submerged into 20–30% sucrose and after embedded in tissue freezing medium (Tissue-Tek Sakura, Netherlands), and finally were frozen in liquid nitrogen. 7 μm thick slices were obtained by utilizing a cryostat (HM 525, Microm, Walldorf, Germany) and stored at −70 °C until use. The samples were stained with hematoxylin–eosin in accordance with the standard procedures for histological evaluation of colonic damage. All tissue sections were examined in an Olympus BH-2 microscope (GMI, MN, USA). Motic Images 2000 software was used for characterization of histopathological changes.

2.8. Assessment of leukocyte involvement

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was assessed de according to the method of Bradley et al. (1982) with slight modifications. Samples were obtained from all animals were weighed and homogenized in 10 volumes of 50 mM potassium phosphate buffer (PBS), pH 7.4. The homogenates were centrifuged at 20,000 × *g*, 20 min, 4 °C. The pellets were again homogenized in 10 volumes PBS, pH 6.0, containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM ethylenediaminetetraacetic acid (EDTA). These homogenates were subjected to one cycle of freezing/thawing and a brief period of sonication.

The supernatants (50 μl) were diluted in 10 volumes of 50 mM PBS, pH 6. Then it was added consecutively 50 μl of O-dianisidine dihydrochloride (0.067%), of HETAB (0.5%) and of hydrogen peroxide (0.003%). Each well containing the complete reaction mixture was incubated for 5 min. The changes in absorbance at 450 nm were measured with a microplate reader (Labsystem Multiskan EX, Helsinki, Finland). One unit MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 U/min in the final reaction volume.

2.9. Assessment of TNF-α and IL-10

Distal colon samples were weighed and homogenized, after thawing, in 10 volumes PBS, pH 7.2, 1% bovine serum albumin (BSA) at 4 °C. They were centrifuged at 12,000 × *g* for 10 min and the supernatants were collected and stored at −80 °C. Mucosal cytokines levels were assayed with quantitative TNF-α (eBioscience, San Diego, CA) and IL-10 (Diaclone, Besançon, France) enzyme immunoassay kits. TNF-α and IL-10 values were expressed as pg/mg tissue.

2.10. Western blot assay

Frozen colonic tissues were weighed and homogenized in ice cold buffer (50 mM Tris–HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM ethylene glycol bis (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), 0.5 mM EDTA, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, 0.01 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 250 mM NaCl). Homogenates were centrifuged (12,000 × *g*, 15 min, 4 °C) and the supernatants were collected and stored at −80 °C. Protein concentration of the homogenate was determined following Bradford's colorimetric method. Aliquots of supernatants containing equal amounts of protein (50 μg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacryamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: COX-2 and iNOS (Cayman Chemical, USA) at dilution of 1:3000, JNK and p-JNK at dilution of 1:2000 and 1:500 respectively (Cell Signalling Technology, USA). Each filter was washed three times for 15 min and incubated with the anti-rabbit immunoglobulin G antibodies (Pierce Biotechnology, IL, USA) and anti-mouse for JNKs. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma–Aldrich, MO, USA). Immunodetection was performed using enhanced chemiluminiscence light-detecting kit (SuperSignal® West Femto Chemiluminescent Substrate, Pierce, IL, USA). Densitometric data were studied following normalization to the control (housekeeping gene). The signals were analyzed and quantified by a Scientific Imaging Systems (KODAK 1D, Image Analysis Software).

2.11. Statistic analysis

All values in the figures and text are expressed as arithmetic means ± standard error of the mean (S.E.M.). Data were evaluated with Graph Pad Prism1 Version 4.01 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Tukey test. *p* values of <0.05 were considered statistically significant. In the experiment involving histology, the figures shown are representative of at least three experiments performed on different days.

3. Results

3.1. Phytochemical screening of butanolic fraction of Abarema cochliacarpos by ESI/MS and NMR ^{13}C characterization

The butanolic fraction of *Abarema cochliacarpos* was characterized by ESI/MS by a high content polyphenols. The tannins are of condensed subgroup, proanthocyanidins, and the major con-

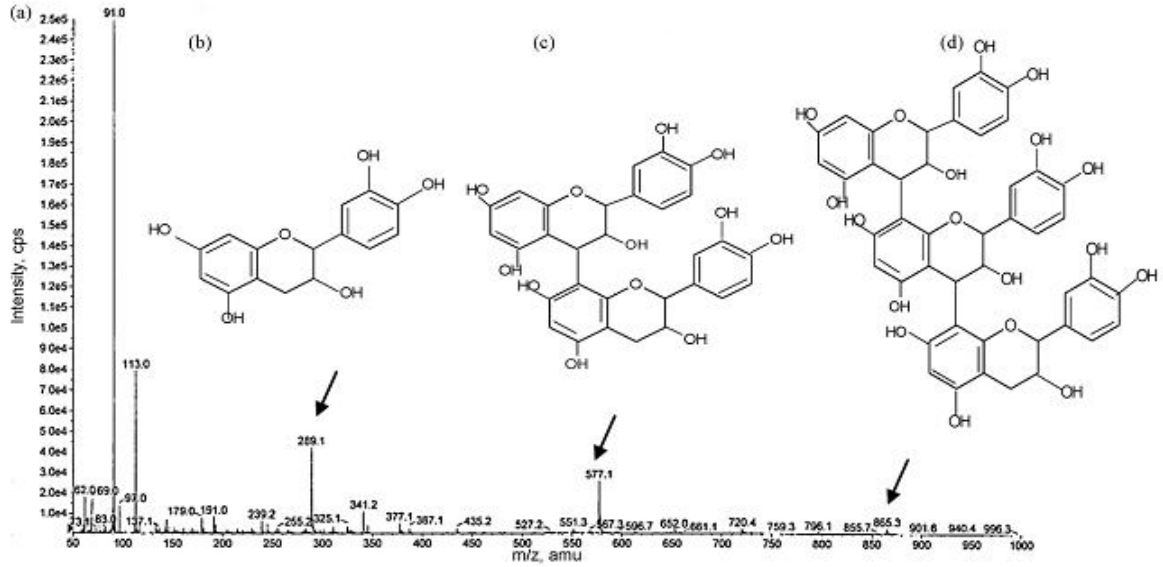

Fig. 1. MS negative mode – pseudomolecular ion [M−H] at m/z 289.1, 577.1 and 865.3. (a) Masses 290 $C_{15}H_{14}O_6$–Flavan-3-ol–Catechin. (b) Masses 578 $C_{30}H_{26}O_{12}$. (c) Masses 866 $C_{45}H_{38}O_{18}$.

stituents were catechins and the minor were dimers and trimers (Fig. 1). The NMR characterization shows the major constituent was (+)-catechins.

3.2. Therapeutic efficacy of butanolic fraction of Abarema cochliacarpos on acute experimental TNBS model

After TNBS intracolonic administration the animals showed prostration, pilorection and hypomotility. The majority of them showed marked body weight loss ($p < 0.001$ vs sham group) accompanied with signs of diarrhoea ($p < 0.05$ vs sham group) (Table 1). After killing, macroscopic inspection of the colon showed a flaccid appearance and evidence of bowel wall thickening, inflammation, ulcers and necrosis. The inflammatory changes of the intestinal tract were associated with a significant increase of weight/length of the colon rats ($p < 0.01$ vs sham group), as an indicator of inflammation, and the presence of adhesions to adjacent organs ($p < 0.001$ vs sham group) (Table 1). Macroscopic lesions in the distal colon were quantified reaching a value of 549.1 ± 96.2 mm² ($p < 0.001$ vs sham group) (Figs. 2A and 3B).

After *Abarema cochliacarpos* treatment (100 and 150 mg/kg) no important variations in clinical symptoms vs TNBS group could be detected (Table 1). However, the highest dose showed significant reduction of adhesions ($p < 0.001$) and the quantification of the macroscopic damage revealed a significant decrease in total gross area of lesion as compared to the TNBS group (279.5 ± 50.6 mm² and 258.5 ± 49.9 mm² respectively, $p < 0.05$) (Figs. 2A and 3C).

3.3. Histological studies of the colon after butanolic fraction of Abarema cochliacarpos treatment on acute experimental TNBS model

Histological study of the colon of sham rats showed a histological normal structure (Fig. 3D). However, slides of TNBS-treated rats presented transmural inflammation involving all layers of the bowel wall. The colonic mucosa was necrotic with destruction of the glands and epithelial cells loss. Extensive granulation tissue with the presence of a massive neutrophilic infiltration, fibroblasts and lymphocytes was also apparent, mainly in the mucosa and submucosa (Fig. 3E). Both doses of *Abarema cochliacarpos* improved the microscopic signs of colitis and preserved some areas of the colonic mucosa structure, remaining intact the epithelium and reducing the inflammatory cells in the lamina propria in comparison with the TNBS group (Fig. 3F).

3.4. Effect of butanolic fraction of Abarema cochliacarpos on colonic leukocyte involvement in TNBS-induced acute colitis

A significant increase in MPO activity, an established marker for inflammatory cell infiltration, also characterized the colitis caused

Table 1
Quantified parameters after administration of butanolic fraction of bark of *Abarema cochliacarpos* (AC, 100 and 150 mg/kg) in rats with acute colitis induced by TNBS intracolonic instillation (10 mg/animal).

Groups	n	Body weight changes (g)	Colon weight/length (g/cm)	Diarrhoea	Adhesions	Mortality (%)
Sham	8	14.31 ± 2.02	0.09 ± 0.002	0	0	0
TNBS	13	−22.30 ± 1.90***	0.15 ± 0.01**	0.70 ± 0.15*	2.80 ± 0.13***	0
TNBS + AC 100	10	−21.60 ± 2.10***	0.14 ± 0.007**	0.60 ± 0.16*	2.60 ± 1.60***	0
TNBS + AC 150	10	−23.60 ± 1.14***	0.14 ± 0.008**	0.40 ± 0.16*	2.20 ± 0.13***	0

Colonic parameters were quantified in the sham group, which received saline instillation. TNBS group received the hapten intracolonically in a vehicle of 50% (v/v) ethanol. Data are expressed as means ± S.E.M.
* $p < 0.05$.
** $p < 0.01$ vs sham.
*** $p < 0.001$ vs sham.
+++ $p < 0.001$ vs TNBS group.

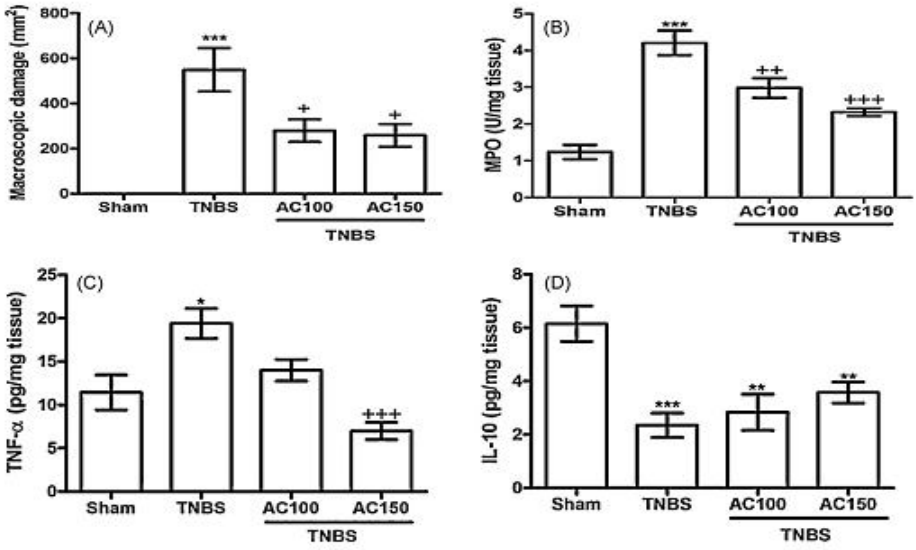

Fig. 2. Effect of acute administration of butanolic fraction of *Abarema cochliacarpos* (AC) on colonic macroscopic damage (A), myeloperoxidase activity (MPO, U/mg tissue) (B), tumor necrosis factor-alpha (TNF-α, pg/mg tissue) (C), and interleukin-10 (IL-10, pg/mg tissue) (D) in trinitrobenzene sulfonic acid-induced colitis model in rats (TNBS, 10 mg/animal). The doses assayed (100 and 150 mg/kg v.o.) significantly decreased the extent and severity of macroscopic damage induced by TNBS instillation. MPO activity and TNF-α levels were increased after TNBS administration compared with sham group. AC administration dose-dependently diminished these parameters. None of the tested doses of AC induced marked changes in IL-10 parameter. The control-sham group received physiological saline instead of the TNBS solution in an equal volume. Data are expressed as the means ± S.E.M. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs sham; $^{+}p < 0.05$; $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs TNBS group.

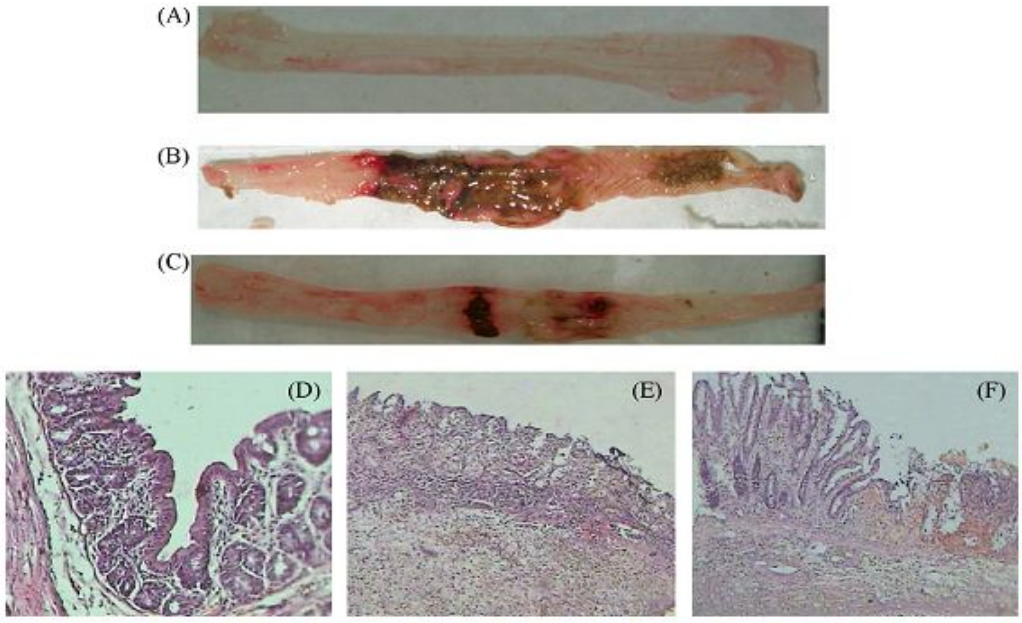

Fig. 3. Effect of acute administration of butanolic fraction of *Abarema cochliacarpos* on colon injury in acute colitis model induced by trinitrobenzene sulfonic acid (TNBS, 10 mg/animal). Representative macroscopic and histological appearance of rat colonic mucosa in control animals (A and D respectively), treated with TNBS (B and E respectively) and butanolic fraction of *Abarema cochliacarpos*-treated animals (150 mg/kg v.o.) (C and F respectively). TNBS lesions were characterized by necrosis of epithelium, focal ulceration and diffuse infiltration of inflammatory cells in the mucosa and submucosa. Treatment with *Abarema cochliacarpos* reduced the morphological alterations associated with TNBS protecting the mucosal architecture. Hematoxylin and eosin. Original magnification 200×.

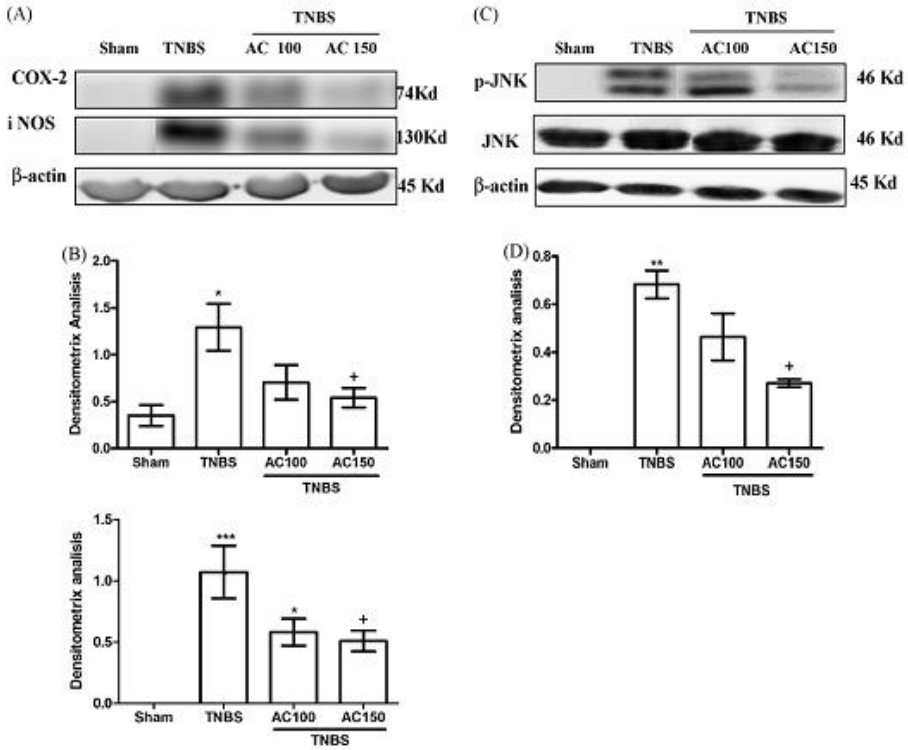

Fig. 4. Effects of acute administration of butanolic fraction of *Abarema cochliacarpos* (AC) on cycloxygenase-2 (COX-2) (A), inducible nitric oxide synthase (iNOS) (B) and activation of the JNK using phosphospecific JNK antibodies after AC administration (C) and administration in trinitrobenzene sulfonic acid (TNBS, 10 mg/animal). COX-2 and iNOS expressions were increased in TNBS group compared with sham animal. AC treatment diminished expression of both proteins. p-JNK is up-regulated in TNBS group and diminished after of AC administration (150 mg/kg). Densitometry was made following normalization to the control (housekeeping gene). The results are representative of three experiments performed on different samples. Data are expressed as the means ± S.E.M. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ vs sham; *$p < 0.05$ vs TNBS.

by TNBS (4.2 ± 0.3 U/mg tissue, $p < 0.001$ vs sham group). Treatment of TNBS rats with the two doses of the extracts, 100 and 150 mg/kg, showed a dose-dependent reduction in the degree of polymorphonuclear neutrophil infiltration (2.9 ± 0.2 U/mg tissue, $p < 0.01$, and 2.3 ± 0.1 U/mg tissue, $p < 0.001$, vs TNBS group respectively) Fig. 2B. These results are consistent with the histological findings (Fig. 3F).

3.5. Effect of butanolic fraction of Abarema cochliacarpos on colonic TNF-α and IL-10 levels in TNBS-induced acute colitis

Colonic injury by acute TNBS administration was also characterized by an increase of the pro-inflammatory Th1 cytokine TNF-α (19.4 ± 1.7 pg/mg tissue, $p < 0.05$ vs sham). After treatment with the two doses of *Abarema cochliacarpos*, was clearly showed a dose-dependent reduction in this cytokine, although only the highest dose (150 mg/kg) significantly modified TNF-α level (6.9 ± 1.0 pg/mg tissue, $p < 0.001$ vs TNBS group) (Fig. 2C).

Respect to anti-inflammatory cytokine IL-10, its production was diminished in rats treated with the hapten vs sham group (2.3 ± 0.4 and 6.1 ± 0.6 pg/mg tissue, $p < 0.001$) and *Abarema cochliacarpos* treatment was not able to significantly enhance its production compared with TNBS group (2.8 ± 0.7 and 3.6 ± 0.4 pg/mg tissue, $p < 0.01$ vs sham group) (Fig. 2D).

3.6. Effect of butanolic fraction of Abarema cochliacarpos on colonic expression of inflammatory proteins in TNBS-induced acute colitis

As regards COX-2 and iNOS expression by Western blotting, exposure of colon to 10 mg/animal of TNBS caused high levels of both proteins in comparison with sham animals ($p < 0.05$ and $p < 0.001$ respectively). *Abarema cochliacarpos* administration induced a down-regulation of both inflammatory proteins, although it was significant at 150 mg/kg only ($p < 0.05$ vs TNBS group) (Fig. 4A and B).

We also examined the expression and activation of JNK by Western blot analysis using phosphospecific antibodies. To standardize protein loading in each line, blots were stripped and re-probed with the corresponding antibodies against both proteins. JNK expression levels remained unchanged in all groups, indicating that JNK protein was constitutively expressed in the colonic tissue and was not significantly changed after TNBS enema or in the presence of the different doses of *Abarema cochliacarpos*. On the other hand, p-JNK protein was significantly increased by TNBS ($p < 0.01$ vs sham group). Nevertheless, oral administration of 150 mg/kg of the extract inhibited the up-regulation of this expression ($p < 0.05$ vs TNBS) (Fig. 4C and D).

4. Discussion

The present study was particularly focused on studying the effects of butanolic fraction of stem bark of *Abarema cochliacarpos* on a TNBS-induced experimental acute colitis, which is considered a model validated to find drugs potentially active in this disease. We have demonstrated that *Abarema cochliacarpos* treatment exhibited amelioration of colonic lesions and histological signs of damage, neutrophil infiltration reduction, proinflammatory cytokine TNF-α diminution and down-regulation of inflammatory COX-2 and iNOS proteins, as well as JNK activation in colonic tissue when compared with TNBS group.

No previous studies on extracts from *Abarema cochliacarpos* bark have been conducted to determinate its anti-inflammatory response at gastrointestinal level, which had been reported in traditional medicine.

In Brazil the name "barbatimão" is most commonly associated with species of genus *Stryphnodendron* (*Stryphnodendron adstringens*, *Stryphnodendron polyphyllum*, *Stryphnodendron obovatum*), which are used for the same therapeutic purposes (Migliatti, 2003), being some of them confirmed by scientific studies. Stem bark extracts of *Stryphnodendron adstringens* have demonstrated significant anti-inflammatory activities (Lima et al., 1998), antiseptic effects (Souza et al., 2007) and antiulcerogenic properties (Audi et al., 1999; Martins et al., 2002). *Stryphnodendron obovatum* and *Stryphnodendron polyphyllum* have also demonstrated a beneficial role in cicatrisation of cutaneous wounds (Lopes et al., 2005). Because of this biological activity, the chemical composition of these species has been investigated (Mello et al., 1996a, 1996b; Lopes et al., 2003) showing that all of them have similar chemical constitutions but not in the content of the total tannins, which may be responsible for the pharmacological activity of this genus. But others species like *Dimorphandra mollis* (Santos et al., 2002) and *Abarema cochliacarpos* (Silva et al., 2006; Silva et al., 2009) are also known as "barbatimão" and are popularly used to treat the same diseases. In many cases, barks of *Stryphnodendron adstringens* are adulterated with these other species (Santos et al., 2002). Thus, it is fundamental the phytochemical and pharmacological study of each "barbatimão" species, defining the profile of activities of each one.

Our phytochemical screening results demonstrated that this extract is rich in flavanols, with high content in catechins monomers, and minor ones dimers and trimers, principally condensate tannins. As we mentioned above catechins are polyphenolic plant metabolite with antioxidant properties, belonging to the flavonoids, which have been evaluated in preclinical IBD models demonstrating promising results (Mazzon et al., 2005; Dryden and Song, 2006) although the mechanisms by which catechins achieve their beneficial effects are still not entirely clear.

Neutrophils infiltration into the inflamed mucosa is one of the most prominent histological features observed in IBD. Activated neutrophils produce reactive oxygen and nitrogen species within intestinal mucosa inducing oxidative stress, which plays a significant role in the pathogenesis of IBD (Martin et al., 2006; Talero et al., 2008). It has been suggested that the main chemoattractants for neutrophils are pro-inflammatory cytokines, such TNF-α, that regulate endothelial molecule expression on vascular endothelial cells and promote neutrophil adherence to these cells. Moreover, TNF-α is released from macrophages in the early inflammatory response playing an important role in TNBS-induced colitis and it is likely the regulator key of the inflammatory cascade in IBD.

Our results showed that MPO activity and TNF-α level were correlated with the development of colonic inflammation and moreover, *Abarema cochliacarpos* administration was able to reduce both parameters and consequently improved colonic damage. These findings are supported by previous *in vitro* and *in vivo* studies where catechins have been shown to reduce the levels of these inflammatory mediators (Soobrattee et al., 2005). In this way Mazzon et al. (2005) showed that catechins from green tea extract remarkably improved the disruption of the colonic architecture and significantly reduced MPO and TNF-α level. In the same lane, Abboud et al. (2008) demonstrated therapeutic effects of epigalocatechin 3-gallate (EGCG), catechin from green tea, attenuating the severity of colitis and significantly reducing the MPO activity as compared with vehicle-treated mice. Other study established that EGCG ameliorated mucosal inflammation by inhibiting TNF-α production, IFN-gamma and NF-kappaBp65 (Ran et al., 2008).

In addition, the degree of inflammation and damage induced by TNBS was paralleled to low levels of the anti-inflammatory cytokine IL-10 in colonic specimens. This finding is in line with a previous study by our group documenting a down-regulation of this cytokine in the colon mucosa in experimental TNBS colitis (Talero et al., 2008). None of the tested doses of *Abarema cochliacarpos* induced marked changes in IL-10 parameter; so the anti-inflammatory effect observed could not be attributed to this mechanism. Some *in vivo* experiments have demonstrated that, for example, green tea did not affect plasma levels of IL-10 in rats subjected to polymicrobial sepsis (Wheeler et al., 2007) or that EGCG did not modify plasma cytokine levels in a mouse model of colitis (Abboud et al., 2008). However, more recently it has been observed that epicatechin was able to enhance IL-10 productions in whole blood stimulated system *in vitro* (Al-Hanbali et al., 2009).

Molecular studies have revealed that phenolics compounds can exert modulatory actions in cell by interacting with a wide spectrum of molecular targets central to the cell signalling machinery. These include down-regulation of pro-inflammatory COX-2 and iNOS enzymes and the activation of MAPKs (Soobrattee et al., 2005).

Many studies have shown that inducible enzymes COX-2 and iNOS are predominantly expressed at sites of inflammation. Increased amounts of these proteins have been found in experimental colitis (Camacho-Barquero et al., 2007; Sánchez-Fidalgo et al., 2007; Talero et al., 2008). In accordance with these previous studies we have also found a strong positive correlation between colitis score and intestinal COX-2 and iNOS expression. Moreover, we have demonstrated by the first time that treatment with the butanolic extract of *Abarema cochliacarpos* significantly reduced both proteins expression in the colonic tissue after acute TNBS administration. Numerous studies have reported that this down-regulation is an important anti-inflammatory mechanism in this disease (Camacho-Barquero et al., 2007; Talero et al., 2008; Sánchez-Calvo et al., 2009).

Different *in vitro* studies have showed that polyphenols including catechins are able to inhibit COX-2 expression. It has been demonstrated how theaflavin monogallate from black tea suppressed COX-2 in human colon cancer cells (Lu et al., 2000). Studies from de Gerhauser et al. (2003) and Hong et al. (2001) have revealed that catechins have COX-1/COX-2 inhibitory activity in different human and mouse cell lines. More recently, a decrease in the levels of COX-2 expression levels in colon cancer cells by a low concentration of EGCG has been reported (Peng et al., 2006).

Respect to NOS modulation, the flavanol theaflavin-3,3'-digallate showed iNOS protein down-regulation in colonic mucosa of TNBS-treated rats (Ukil et al., 2006). In other anti-inflammatory model, a neuroprotective effect of EGCG was observed in part, due to modulation of iNOS expression (Sutherland et al., 2005). Moreover, EGCG decreased the activity and protein levels of iNOS in lipopolysaccharide (LPS)-activated peritoneal macrophages (Lin and Lin, 1997).

In addition JNK pathway is considered to be a potentially relevant target for therapy inflammatory disease states. JNK regulates the maturation and activity of T cells and synthesis of pro-inflammatory cytokines such as IL-2, IL-6 and TNF-α (Roy et al., 2008). Moreover, it has been reported that TNF-α signalling is linked to activate MAPKs.

JNK activation has been recently demonstrated in an experimental model of IBD (Sánchez-Calvo et al., 2009) and in patients with this disease (Mitsuyama et al., 2006). In the same way, Assi et al. (2006) showed a significant reduction in histological damage scores and macrophage infiltration in mice with DSS-induced colitis and treated with the JNK inhibitor SP600125. According to these findings, our results showed that the butanolic fraction of *Abarema*

cochliacarpos was able to inhibit JNK activation improving disease progression.

5. Conclusion

In conclusion, we have shown for the first time, that the extract obtained from steam bark of *Abarema cochliacarpos* possesses active substances, which exert marked protective effects in acute experimental colitis mainly by anti-inflammatory mechanism. These results confirm and justify, at least in part, the popular use of this plant to treat gastrointestinal diseases. However, a detailed study should be carried out in order to continue elucidating the phytochemical compounds of bark of *Abarema cochliacarpos* and further evaluate its protective effect in this context.

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Artigo 3 – Artigo em preparação

Título: Chronic administration of *Abarema cochliacarpos* attenuates the TNBS-induced colitis in rats

1 Introduction

The inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC) are illness characterized by a chronic clinical course of relapse and remission associated with self-destructive inflammation of the gastrointestinal tract. What differentiates IBD from the inflammatory responses seen in normal gut is an inability to downregulate these responses (Gambero *et al.,* 2007). One of the earliest factors involved is the breach of the intestinal epithelial barrier and the development of abnormal immune and inflammatory responses that are mediated predominantly by activated neutrophils, monocytes, lymphocytes, and macrophages (Schmidt & Stallmach, 2005; Thompson-Chagoyan et al., 2005). Infiltrated granulocytes and macrophages produce high levels of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), that plays a central role in mucosal inflammation and is likely to be at the apex of the inflammatory cascade in CD (Waetzig et al., 2002, Talero et al., 2008). Another mediators that have been closely associated with the initiation and maintenance of intestinal inflammation in IBD are COX-2 and i-NOS, which play a pivotal role in mediating inflammation. COX-2 activation produces excessive PGE_2 and TXB_2, which are important inflammatory mediators that contribute to intestinal hyperemia, edema, and even dysfunction and iNOS activation leads to excessive production of NO, which may be detrimental to the integrity of the colonic mucosal (Dignass et al.,1995, Sánchez-Calvo et al., 2009).

Among the signalling pathways who are involved in the development or propagation of IBD, the nuclear factor κB (NF-κB) and/or the mitogen-activated protein kinases (MAPK) are of particular interest (Desreumaux, 2004), because thus modulating a number of different steps in the inflammatory cascade. These include production of pro-inflammatory cytokines (tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, interferon gamma (IFN-γ), IL-12, and IL-6) in different cell-types, the expression of receptors essential for neutrophils activation and chemotaxis and certain proteins, important determinants of colonic damage, i.e. cyclo-oxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) (Waetzig et al , 2002, Hollenbach et al , 2005).

Among MAPKs, recent studies have highlighted the importance of the JNK pathway in IBD. Through the signaling pathways inflammatory responses – mainly cytokine production and inflammatory cell infiltration- have been shown to be attenuated by usage of specific inhibitors against p38 and JNK (Kwon et al., 2007; Garat & Arend, 2003; Di Mari et al.,2007 ; Hollenbach et al., 2005, Assi et al., 2006; Broom et al., 2009). Much interest has centred on the beneficial effects of flavanols, such catechins in IBD. Importantly, the cytoprotective action of these compounds was found to involve both the inhibition of caspase-3 activation and activation of pro-apoptotic MAPK proteins (Willians et al., 2004).

In recent years, flavonoids have been more progressively used as a natural ingredient in beneficial health products. This group of compounds is divided into six major subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins). Much interest has centred on the beneficial effects of flavanols, and there is growing evidence that at the cytoprotective nature of these polyphenols is based on their interactions within signalling pathways (Sutherland, 2005).

Abarema cochliacarpos (Gomes) Barneby & Grimes, Mimosaceae, is an ornamental tree and native medicinal species in to Brazil. It in popularly known as "barbatimão" or "babatenã". It appears mainly in the Atlantic Forest and in the c*aatinga* (dryland) region of Northeastern Brazil, which hosts many different ethnic communities, (Albuquerque et al., 2007; World Conservation Center, 1998). In this same region, on the traditional medicine of Colônia Treze village (Lagarto/Sergipe) the decoction of stem bark of *A. cochliacarpos* is frequently used for wound-healing, as an analgesic and anti-inflammatory (Silva et al., 2006; Silva et al., 2009).

Previous pharmacological studies were performed by our group to test the effect of the *A. cochliacarpos* stem bark. The antiulcer effect of the aqueous extract of the bark was tested in experimental alcohol gastric ulcers demonstrating for the first time a reduction of the lesions (Silva, et al., 2006). Recently, the butanolic fraction of the methanolic extract (AC) 150mg/kg was tested on acute experimental colitis mode induced by trinitrobenzene sulfonic acid (TNBS) and showed anti-inflammatory effects (da Silva et al., 2010). The phytochemical screening of this fraction was characterized by electrospray ionization (ESI)/MS by high content of polyphenols. Its major constituents were catechins, flavonoid-type compounds and the minor were its dimers and trimers. The tannins belong to the proanthocyanidin subgroup, The NMR characterization shows the major constituent was (+)-catechins (da Silva et al., 2010).

Ethnopharmacological studies showed that *Abarema cochliacarpos* is used in traditional medicine for many diseases, including gastrointestinal diseases, through the daily ingestion of this decoction or the tincture, which is made by placing the bark in white wine or "cachaça" (Silva et al., 2006, Santos, 2008). Thus, considering this fact, and the promising results that have been showed by butanolic fraction of methanolic extracts of *Abarema cochliacarpos* (AC 150 mg/kg), rich catechins and tannins, in the acute colitis model induced by TNBS, we decided to assess the chronic response associated with AC 150 mg/kg after 14 consecutive days administration in experimental colitis induced by TNBS.

We have assessed the inflammatory response by histological analysis as well as by the determination of inflammation markers such as MPO activity and cytokines profile. In order to gain a better insight into the action mechanism(s) of the observed protective effects of AC150, the expression of inducible enzymes COX-2 and iNOS by Western blotting, and the expression and activation of JNK have also been analyzed.

2 Materials and Methods

2.1 Plant material

The bark of *A. cochliacarpos* was obtained from collection by Maria Silene da Silva and the folk medicine practitioner, in Colônia Treze village, Lagarto city, Sergipe state, Brazil, in July 2006. Voucher specimens of this species were identified by Prof. Dra. Christine Niezgoda from Field Museum of Natural History and was deposited under number 007628 at the Federal University of Sergipe.

2.2. Preparation of the extract

Fresh bark of *A. cochliacarpos* was dried at room temperature for 10-14 days. Then it was powdered (1.200 g) and exhaustively extracted with methanol (5L) at room temperature during 15 days at room temperature by maceration. The suspension was concentrated at 40º C under reduced pressure to provide a crude metanolic extract with 34.61% yield (415.4 g).

The methanolic extract (200.0 g) was partitioned three times with a mixture of ethyl acetate: water (1:1, v/v). The ethyl acetate fraction was evaporated at 35°C under reduced pressure

affording an ethyl acetate fraction of 50.12 g. The aqueous phase was partitioned with a mixture of *n*-butanol: water (1:1 v/v, 3 times), affording 99.69 g of butanolic extract fraction and 50.19 g of aqueous fraction.

2.3 Animals

Male and female Wistar rats supplied by Animal Services of the University of Seville, Spain, weighing 180–200 g, were placed in singled in cages with wire-net floors in a controlled room (temperature 24–25^{O} C, humidity 70–75%, lighting regimen of 12L/12D) and were fed a normal laboratory diet (Panlab, Barcelona, Spain). Rats were deprived of food for 12 h prior to the induction of colitis, but were allowed free access to tap water throughout. They were randomly assigned to groups of 8–13 animals. Experiments followed a protocol observed by the Animal Ethics Committee of the University of Seville and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EC).

2.4 Induction of colitis

Colitis was induced according to the procedure described by Morris et al. (1989). Briefly, rats were slightly anesthetized with 12% chloral hydrate by intraperitoneal route (i.p.) following a 12 h fast, and then a medical-grade polyurethane cannula for enteral feeding (external diameter 2mm) was inserted into the anus and the tip was advanced to 8 cm proximal to the anus verge. TNBS (Sigma-Chemical Co., St. Louis, MO, USA) dissolved in ethanol (50%, v/v) was instilled into the colon through the cannula (TNBS-30 mg in a volume of 0.25 ml to induce a long-chronic colitis). AC 150 mg.kg^{-1} was administered 24 h after TNBS instillation and daily during the 2 weeks before the sacrifice. A reference control group was applied for comparison with the TNBS colon instillation group: the Sham group that received physiological saline instead of the TNBS solution in a comparable volume. Sham and TNBS groups also received the vehicle (2% Tween 80 in a volume of 10 mL/kg body weight) by v.o. route.

Following the instillation of the hapten, the animals were maintained in a head-down position for a few minutes (2–3 min) to prevent leakage of the intracolonic instillate. Butanolic fraction of *Abarema cochliacarpos* (150 mg.kg^{-1}) was suspended in 2% Tween 80 (Sigma-Chemical

Co., St. Louis, MO, USA) (Santos el al, 2004) and was administered in a volume of 10 mL/kg body weight by oral route. The rats were checked daily for behavior, body weight, and stool consistency.

2.5 Assessment of colitis

An independent observer who was blinded to the treatment evaluated the severity of colitis. For each animal, the distal 10 cm portion of the colon was removed and cut longitudinally, slightly cleaned in physiological saline to remove fecal residues and weighed. Macroscopic damage was quantified measuring the extent of the lesions in the distal colon (mm^2) using Bioview 4 (AvSoft, Brazil) an image analysis software (Khun, 2004). The presences of adhesions (score 0–2), and/or stool consistency (score 0–1) were evaluated according to the criteria of Bobin-Dubigeon et al. (2001) with slight modifications. Photographs taken from colon samples were digitized using Kodak D290 Zoom camera (Eastman Kodak Co., Rochester, NY, USA). Pieces of colon were collected and frozen in liquid nitrogen for measurement of biochemical parameters.

2.6 Histological studies

For examination with the light microscope we used tissue samples from the distal inflamed colon of each animal and fixed in 4% buffered formaldehyde, then submerged into 20-30% sucrose and after embebed in tissue freezing medium (Tissue-Tek Sakura, Netherlands), and finally were frozen in liquid nitrogen. 7 µm thick slices were obtained by utilizing a cryostat (HM 525, Microm, Walldorf, Germany) and stored at -70° C until use. The samples were stained with hematoxylin–eosin in accordance with the standard procedures for histological evaluation of colonic damage. All tissue sections were examined in an Olympus BH-2 microscope (GMI, MN, USA). Motic Images 2000 software was used for characterization of histopathological changes.

2.7 Assessment of leukocyte involvement

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was assessed de according to the method of Bradley et al. (1982) with slight modifications. Samples were obtained from all animals were weighed and homogenized in 10 volumes of 50 mM potassium phosphate buffer (PBS), pH 7.4. The homogenates were centrifuged at 20,000*g*, 20 min, 4°C. The pellets were again homogenized in 10 volumes PBS, pH 6.0, containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM

ethylenediaminetetraacetic acid (EDTA). These homogenates were subjected to one cycle of freezing/thawing and a brief period of sonication.

The supernatants (50 µl) were diluted in 10 volumes of 50 mM PBS, pH 6. Then it was added consecutively 50 µl of O-dianisidine dihydrochloride (0.067%), of HETAB (0.5%) and of hydrogen peroxide (0.003%). Each well containing the complete reaction mixture was incubated for 5 min. The changes in absorbance at 450 nm were measured with a microplate reader (Labsystem Multiskan EX, Helsinki, Finland). One unit MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 U/min in the final reaction volume.

2.8 Assessment of TNF-α and IL-10

Distal colon samples were weighed and homogenized, after thawing, in 10 volumes PBS, pH 7.2, 1% bovine serum albumin (BSA) at 4°C. They were centrifuged at 12,000 *g* for 10 min and the supernatants were collected and stored at -80°C. Mucosal cytokines levels were assayed with quantitative TNF-α (eBioscience, San Diego,CA) and IL-10 (Diaclone, Besançon,France) enzyme immunoassay kits. TNF-α and IL-10 values were expressed as pg/mg tissue.

2.9 Western blotting assay

Frozen colonic tissues were weighed and homogenized in ice cold buffer (50 mM Tris–HCl, pH 7.5, 8mM $MgCl_2$, 5mM ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), 0.5 mM EDTA, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, 0.01 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 250 mM NaCl. Homogenates were centrifuged (12,000 x g, 15min, 4°C) and the supernatants were collected and stored at -80°C. Protein concentration of the homogenate was determined following Bradford's colorimetric method. Aliquots of supernatants containing equal amounts of protein (50 µg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacryamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: COX-2 and i-NOS (Cayman Chemical, USA) at dilution of 1:3000, and JNK and pJNK at at dilution of 1:2000 and 1:500, respectively) (Cell Signalling Technology. USA). Each filter was washed three times for 15 min and incubated with the anti-rabbit immunoglobulin G antibodies (Pierce Biotechnology, IL,U.S.A) and anti-mouse for JNK. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma-Aldrich, MO, USA).

Immunodetection was performed using enhanced chemiluminiscence light-detecting kit (SuperSignal® West Femto Chemiluminescent Substrate, Pierce, IL, USA). Densitometric data were studied following normalization to the control (housekeeping gene). The signals were analyzed and quantified by a Scientific Imaging Systems (KODAK 1D, Image Analysis Software).

2.10 Statistic analysis

All values in the figures and text are expressed as arithmetic means ± standard error of the mean (S.E.M.). Data were evaluated with Graph Pad Prism1 Version 5 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Tukey test. p values of <0.05 were considered statistically significant. In the experiment involving histology, the figures shown are representative of at least three experiments performed on different days.

3 Results

At 14 days after intracolonic administration of TNBS, the control animals underwent severe anorexia with a marked body weight loss and intense diarrhea in the majority of animals. Presence of adhesions to adjacent organs and a marked increase in the weight/length of the colon were also observed in TNBS treated rats in comparison with sham group ($p<0.001$) (Table 1). Macroscopic inspection of the colon obtained two weeks after colitis induction showed a flaccid appearance and evidence of bowel wall thickening, inflammation and ulcers. The damage was quantified and the affected area was 1169±116.30 mm^2 ($p<0.001$) (Figure 1).

In the treatment of TNBS-rats with AC 150, with respect to the presence of adhesions to adjacent organs and weight/length of the colon, significant changes were observed in the assay period ($p<0.05$ and $p<0.01$ vs. TNBS group, respectively). The butanolic fraction was able diminished diarrhea in this dose assay ($p<0.05$, vs TNBS), although no significant reduction in the body weight loss was observed. Treatment with AC 150 significantly ameliorated the extent and severity of the colonic inflammation, reflecting signs of greater recovery as evidenced by the quantification of macroscopic damage (291.8±10.82 mm^2; $p<0.001$ vs. TNBS group).

The histological analysis of the colon of sham-treated rats showed typical features of normal structure (Fig. 2D). In TNBS-treated rats, the inflammation extended through the mucosa

and submucosa. Extensive granulation tissue with presence of fibroblasts and lymphocytes, leukocytes, and diffuse inflammatory infiltrates was apparent (Fig. 2E). After administration of AC 150, the colonic histopathology was dramatically reduced: there was an attenuation of morphological signs of cell damage, the colonic mucosa showed ulcers in the process of healing, evolution to a more chronic inflammatory infiltrate, with mononuclear predominance and initiation of a repair process. The mucosa showed a healing course with signs of mucosal reepithelization (Fig. 2F, 2G).

As shown in Figure 1, the colitis caused by TNBS was also characterized by a marked increase in MPO activity, an indicator of the infiltration of the colon with polymorphonuclear leukocytes. This result was consistent with the histological findings. The study of MPO activity showed that AC 150 significantly reduced the degree of polymorphonuclear neutrophil infiltration as compared to TNBS group (0,54±0.16 U/mg tissue, $p<0.05$ vs. TNBS group).

Regarding the proinflammatory cytokines production, the colonic injury provoked by 30 mg/animal of TNBS administration was characterized by a significant increase in TNF-α levels (40.95±5.45 pg/mg tissue, $p<0.01$). After treatment with AC 150 mg/kg/day, data clearly indicated a significant reduction in this cytokine (18.94 ± 0.44 pg/mg tissue, $p<0.01$, vs. TNBS group) (Figure 1C).

However, levels of IL-10 were diminished in TNBS (2.05±0.71 pg/mg tissue, $p< 0.05$ vs. sham group). In contrast, the AC 150 increased IL-10 levels, reaching values similar to sham group (6.63± 0.70 pg/ mg tissue, $p < 0.01$ vs. TNBS group) (Figure 1D).

The levels of expression of COX-2 and i-NOS were measured by western blotting of cytosolic extracts from colonic mucosa. Exposure of colon to TNBS caused strong expression of COX-2 and i-NOS, indicating that these proteins expression could be induced at the chronic stage of colonic lesion caused by TNBS. Nevertheless, oral administration of AC150 was able to diminish the up-regulation of both COX-2 and i-NOS proteins ($p<0.05$, vs TNBS), (Figure 3A, 3B).

Phospho-JNK protein was not detected in cytosolic extracts of normal colon mucosa, whereas a high expression appeared in colon mucosa from control TNBS-treated rats. Nonetheless, upon treatment with AC150 the protein expression of pJUNK was decreased ($p<0.05$ vs TNBS). As shown in this figure 3C, the levels of JUNK protein remained unchanged in all groups, indicating that JUNK protein was constitutively expressed in the colonic tissue and was not significantly changed after TNBS-enema or in the presence of AC150.

4 Discussion

In particular we have demonstrated that, after 14 consecutive days, AC150 treatment administration inhibits the degree of adhesions, diarrhea and colon weight/length, and also the degree of colonic injury. There was an attenuation of morphological signs of cell damage, the colonic mucosa showed ulcers at the beginning of reepithelization and healing process. The polymorphonuclears infiltration in the colon, and the increased expression of TNF-α production caused by TNBS also was diminished after AC150 treatment, although anti-inflammatory IL-10 levels was increased. Others parameters associated with the intestinal inflammation also was diminished by AC150, as the levels phosphorylation of JNK and down-regulation of inflammatory COX-2 and iNOS proteins.

Our phytochemical screening results characterized this extract and demonstrated that have (+)-catechins is its main bioactive constituents. Since the intake of catechins has been associated with a wide variety of beneficial health effects, as demonstrated by several studies, including the experimental model of ulcerative colitis (da Silva et al. 2010, Sato et al., 1998; Mazzon et al., 2005) and since (+)-catechins are the most abundant polyphenols of the butanolic fraction of the *Abarema cochliacarpos*, we have studied its therapeutic effects on TNBS-induced experimental chronic colitis model.

The studies of bioavailability of catechins show that most of an oral dose of catechins remains in the gut and becomes available for metabolism by the colonic microflora, some certainly is absorbed and appears in the plasma (Gee and Johnson, 2001; Nakagawa and Miyazawa, 1997). Studies by Okushio *et al.* (1996) have confirmed that the principal catechins of green tea are absorbed in the rat small intestine by measuring their appearance in the portal circulation after an oral dose. All these observations may have significance on the beneficial effect of administration of AC150 in ulcerative chronic colitis.

Whatever the trigger of IBD, neutrophils are early responders to all types of insult and play a central role in the inflammatory process. During the initial innate immune response, they are seen passing from the circulation through gaps in the vascular endothelium to infiltrate the tissues (Villegas et al., 2003). Once there, neutrophils release antimicrobial peptides, as enzyme MPO, which catalyses the formation of such potent cytotoxic oxidants as hypochlorous acid from hydrogen peroxide and chloride ions and N-chloramines (Martin et al., 2006) and reactive oxygen

intermediates that may in themselves cause further tissue damage (Hanauer, 2006).The increase in MPO activity in TNBS-treated animals was reduced after chronic administration of AC 150 during 14 days. Microscopic evaluation also revealed an increased number of neutrophil leukocytes that possibly contributed to the elevated MPO activity observed in 30 mg/kg TNBS-induced colitis.

The antioxidant activity of catechins has been demonstrated for many studies. *In vitro* studies by Sobratee et al. (2005) showed the highest percentage inhibition of deoxyribose degradation due to $OH^{\bullet}$ scavenging of (+) catechin, and its strong scavengers of anion hypochlorite (ClO^-) in the hypochlorite scavenging assay. Is this way, the reduction in colonic MPO activity, in the according the histological finding of the reduction cellular infiltration after treatment with AC150 may be reflective of its antioxidant and anti-inflammatory effects of its constituents in the prevention of this model of experimental colitis.

Many works by our group showed that in this well-characterized hapten reagent TNBS-induced colitis model increased levels of mucosal proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, induce the production of chemoattractants for neutrophil, which regulate endothelial adhesion molecule expression (ICAM-1) on vascular endothelial cells and promote neutrophil adherence to these cells (Sánchez-Fidalgo, et al., 2007; Camacho-Barquero et al., 2007; Talero et al., 2008; Sánchez-Calvo et al., 2009).

On the other hand, the anti-inflammatory effects of IL-10 citokine inhibit both antigen presentation and subsequent release of pro-inflammatory cytokines, thereby attenuating mucosal inflammation in IBD (Sanchez-Muñoz et al., 2008). An inactivation of IL-10 in mice results in an increased production of IL-12 and IFN-γ (Schreiber et al., 1995). The pivotal role played by IL-10 within the mucosal immune system has been extensively studied in the chronic ileo-colitis that develops in gene-targeted IL-10 knockout mice and by its therapeutic efficacy in several animal models of colitis (Wirtz & Neurath, 2007).

The present results show that AC150 chronic administration was able significantly to diminish TNF-α levels, but higher IL-10 levels in the animals treated with this fraction. The increased levels of TNF-α and IFN-γ in the rat's model revealed an important role for immune dysregulation and altered cytokines patterns that are similar to the human disease (Ran et al., 2008). Recents studies show a down-regulation of IL-10 cytokine in a chronic experimental colitis in TNBS-treated rats (Camacho-Barquero et al., 2007; Sánchez-Fidalgo, et al., 2007; Talero et al., 2008; Sánchez-Calvo et al., 2009). The present study is in accordance with this suggestion. Thus,

AC150 application seems capable of tilting by dampening the Th1 response. Therefore, this cytokine shift may be the mechanism of action of this fraction responsible for reversing TNBS – induced colitis in rats.

Many studies show similar effects of green tea extracts, rich epicatechins, in diminish TNF-α activity in others models of IBD (Mazzon et al., 2005, Ran et al.,2008, Varilek et al.,2001), and it augment the IL-10 levels in by human leukocytes *in vitro* (Crouvesier et al., 2008).

In the present study, we have also demonstrated that macroscopic damage was associated with both COX-2 and iNOS overexpression, and the AC 150 fraction of *A. cochliacarpos* exerts down-regulation effects to inflammatory proteins in the TNBS colitis model. Many reseachers show that treatment with other catechins types, as the epicatechin gallate, resulted in a decrease in COX-2 mRNA expression in HCA-7 and HT-29 cells (Peng et al., 2006). *In vivo*, treatment with these agents significantly attenuates the inflammation-related mouse colon carcinogenesis by reducing the expression of COX-2 protein and mRNA (Shirakami, et al 2008). In ciclooxigenase enzyme inhibitory assay (+)-catechins have showed COX-1/COX-2 inhibitory activity (Seeram et al., 2003). Studies have also reported the scavenge by $NO^{\bullet}$ and peroxynitrite catechins inhibit the excessive production of $NO^{\bullet}$ by the inducible form of nitric oxide synthase (Chan et al., 1997; Paquay et al., 2000). *In vivo*, catechins reduced the contents of NO in acid acetic model induced colitis (Ran et al., 2008). Therefore, this down regulation activity of both COX-2 and iNOS expression by AC150 could be assigned to (+)-catechins effects present in this fraction

Additionally, iNOS acts in synergy with COX-2 to promote intestinal inflammation Furthermore, both COX-2 and i-NOS expressions are upregulated by MAPK in intestinal epithelial cells (Itzkowitz, 2006). The potential modulation of MAP kinase signalling pathways by flavonoids is significant, as ERK1/2 and JNK are involved in growth factor-induced mitogenesis, differentiation, apoptosis and various forms of cellular plasticity (Rice-Evans et al., 1995; Yua &Yanker, 2000; Herdegen et al., 1997).

The JNK pathway is considered to be a potentially relevant target for therapy inflammatory disease states. JNK regulates the maturation and activity of T cells and synthesis of pro-inflammatory cytokines such as interleukin-2 (IL-2), IL-6 and TNF-α (Roy et al., 2008).

In our study, chronic inflammatory conditions induced by TNBS were accompanied by the presence of detectable quantities of P-JNK in the citosolic extracts whereas this was decreased upon treatment with AC 150 to rats. This findings are in line with a study by Lu et al. (1998) which

demonstrated that the level of phosphorylated JNK1 was reduced by catechins in vascular smooth muscle cells; phosphorylated ERK1 and ERK levels, on the other hand, were not. In this same study, direct measurement of kinase activity by immune complex kinase assay confirmed that JNK1 activity was inhibited by epigallocatechin treatment. These results demonstrate that epigallocatechin preferentially reduced the activation of JNK/SAPK (stress-activated protein kinase) signal transduction pathway.

In conclusion, this study demonstrates that the degree of colitis caused by chronic administration of TNBS is significantly attenuated by AC150. The anti-inflammatory effects are associated with a reduction in (i) proinflammatory T-helper 1 cytokine response and an increase in (ii) upregulation of antiinflammatory T-helper 2 cytokine response leading to the attenuation of the recruitment of neutrophils, attenuation of COX-2 and iNOS protein expression and potential modulation of JNK signaling pathway (iii) and ultimately (iv) tissue injury. The results suggest a significant protective effect after the chronic administration of *Abarema cochliacarpos* in the chronic phase of inflammatory response associated with the TNBS-induced colitis.

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Table 1

Quantified parameters after administration of butanolic fraction of bark of *A. cochliacarpos* (AC, 150 mg/kg) in rats with chronic colitis induced by TNBS intracolonic instillation (30 mg/animal).

Group	**n**	**Body weight**	**Adhesions (score 0–2)**	**Diarrhea (score 0-1)**	**Colon weight/lenght (g/cm)**	**Mortality (%)**
Sham	8	180.25±3.14	0	0	0.09±0.004	0
TNBS-30	8	163.40±3.19***	1.80±0.20***	0.83±0.16***	0.56±0.09***	40
AC150	8	172.23±2.93	1.20±0.20+	0.25±0.25+	0.29±0.03++	20

Colonic parameters were quantified in the sham group, which received saline instillation. TNBS group received the hapten intracolonically in a vehicle of 50% (v/v) ethanol. Data are expressed as mean±S.E.M. (***)$p<0.001$ vs. Sham; (+) $p<0.05$ and (++)$p<0.01$ vs. TNBS

Figure 1

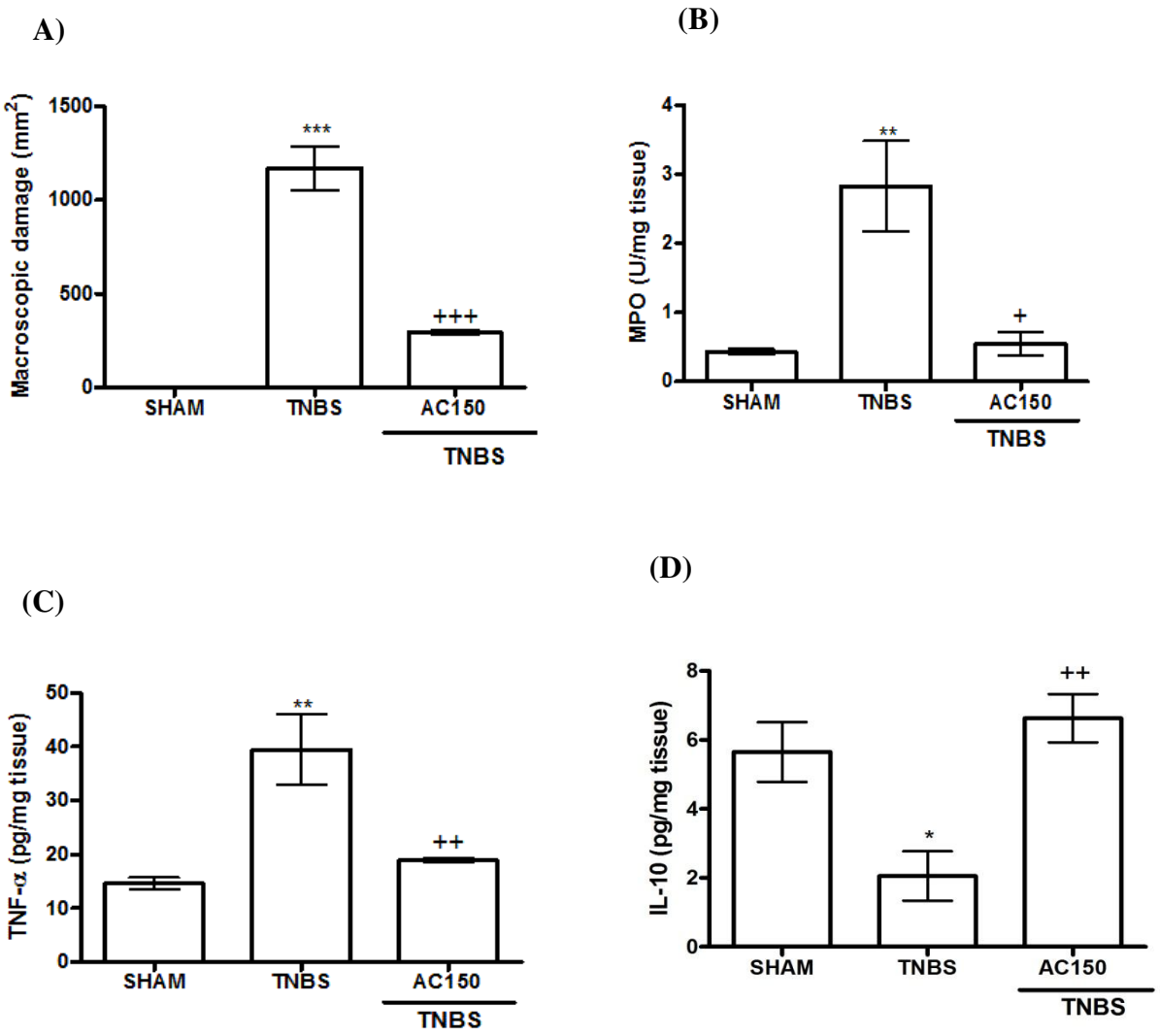

Fig. 1 - Effect of chronic administration of butanolic fraction of Abarema cochliacarpos (AC) on colonic macroscopic damage (A), myeloperoxidase activity (MPO, U/mg tissue)(B), tumor necrosis factor-alpha (TNF-α, pg/mg tissue) (C), and interleukin-10 (IL-10, pg/mg tissue) (D) in trinitrobenzene sulfonic acid-induced colitis model in rats (TNBS,30 mg/animal). The dose assayed (150 mg/kg v.o.) significantly decreased the extent and severity of macroscopic damage induced by TNBS instillation. MPO activity and TNF-α levels were increased after TNBS administration compared with sham group. AC administration dose-dependently diminished these parameters, and was able in augment IL-10 parameter. The control-sham group received physiological saline instead of the TNBS solution in an equal volume. Data are expressed as the means±S.E.M. One way ANOVA followed by Tukey's test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs sham; +$p < 0.05$; ++$p < 0.01$ and +++$p < 0.001$ vs TNBS group,

Figure 2

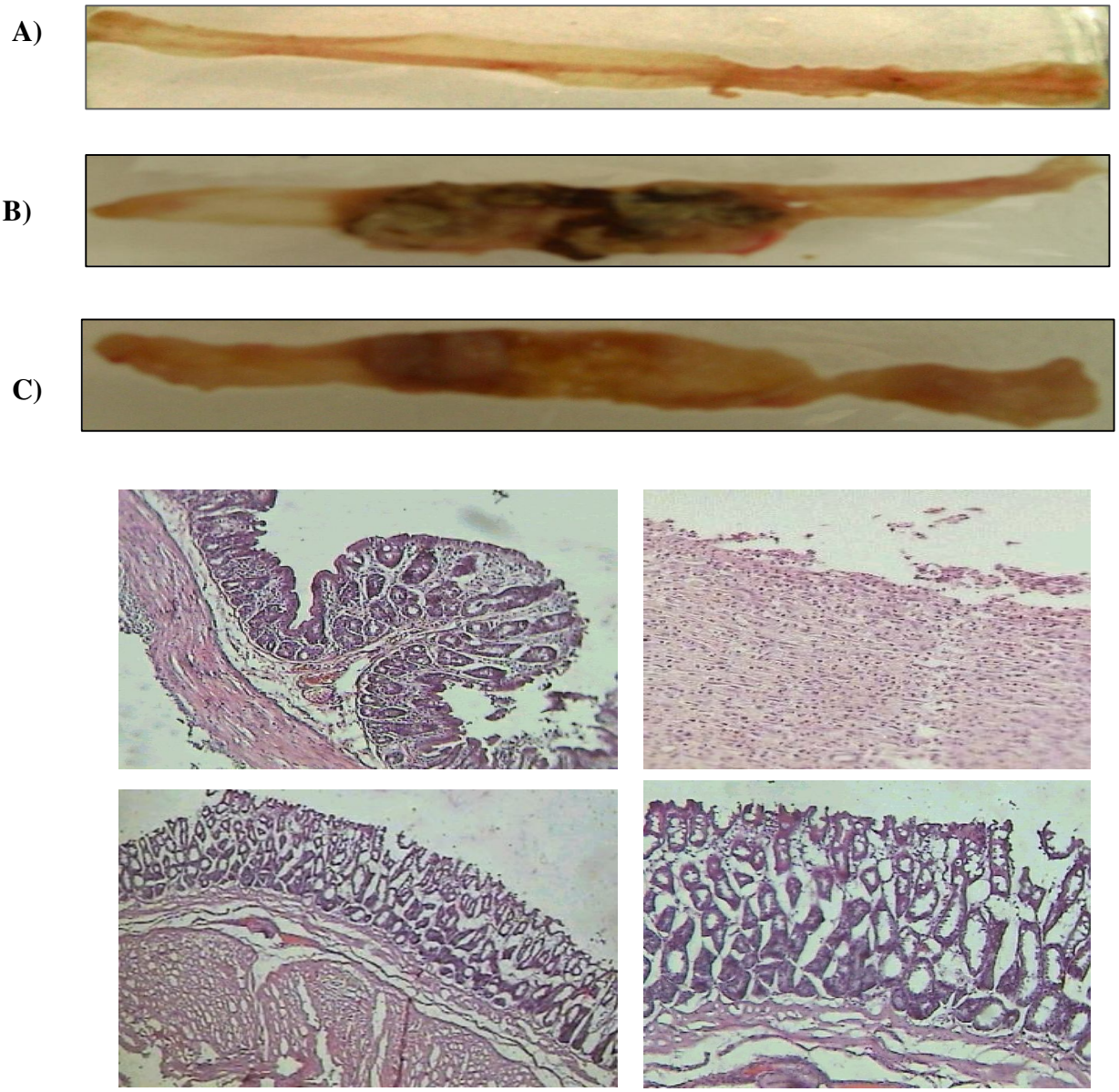

Fig. 2 -Effect of butanolic fraction of *A. cochliacarpos* on colon injury in chronic colitis model induced by trinitrobenzene sulfonic acid (TNBS, 30 mg/animal). Representative macroscopic and histological appearance of rat colonic mucosa in control animals (A and D respectively), TNBS treatment (B and E respectively) and butanolic fraction of *A. cochliacarpos*-treated animals (150 mg kg^{-1} v.o.) (C, F and G respectively). TNBS lesions were characterized by necrosis of epithelium, focal ulceration and diffuse infiltration of inflammatory cells in the mucosa and submucosa. Treatment with *A. cochliacarpos* reduced the morphological alterations associated with TNBS protecting the mucosal architecture. Hematoxylin and eosin. Original magnification: D, E and F – 20X; G, 100X

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Figure 3

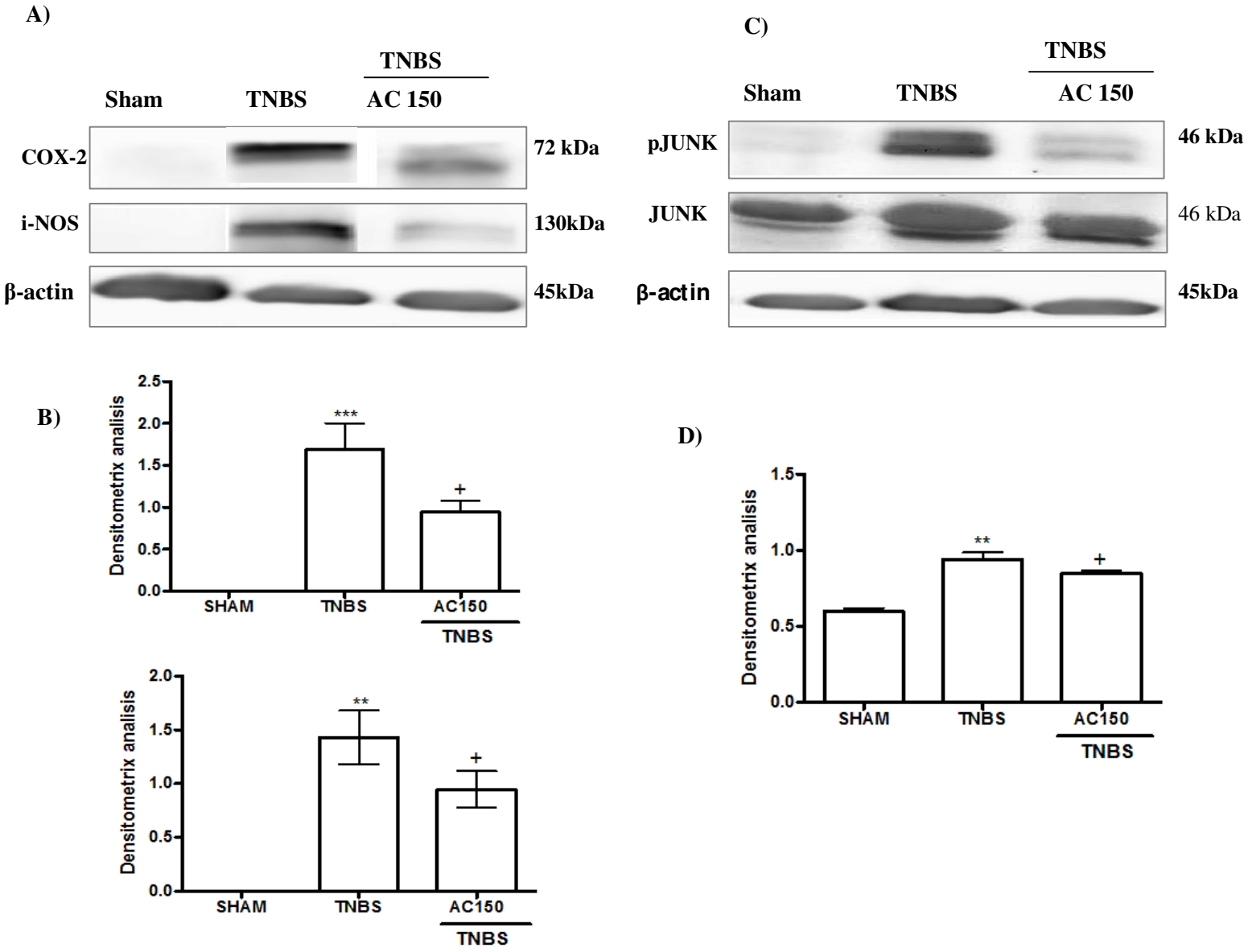

Fig. 3. Effects of chronic administration of butanolic fraction of *Abarema cochliacarpos* (AC150 mg/kg) on cycloxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) (A and B) and activation of the JNK using phosphospecific JNK antibodies after AC administration (C and D) and administration in trinitrobenzene sulfonic acid (TNBS, 30 mg/animal). COX-2 and iNOS expressions were increased in TNBS group compared with sham animal. AC treatment diminished expression of both proteins. p-JNK is up-regulated in TNBS group and diminished after of AC administration. Densitometry was made following normalization to the control (housekeeping gene). The results are representative of three experiments performed on different samples. Data are expressed as the means±S.E.M. One way ANOVA followed by Tukey's test. **p < 0.01 and ***p < 0.001 vs sham; +p < 0.05 vs TNBS.

V Considerações Finais

Atividade Antiulcerogênica Gástrica

Triagem dos extratos e frações de *Abarema cochliacarpos*

Abarema cochliacarpos	**Modelo, Rota e período administração**	**Parâmetros**
Extrato CE (100, 200 e 400 mg/kg)	Etanol, v.o. 1 h antes do etanol	↓ Área de lesão
Extrato ME (100, 200 e 400 mg/kg)	Etanol, v.o. 1 h antes do etanol	↓ Área de lesão
Partição do Extrato ME: AF (12.50, 25, 50, 100, 150 e 200 mg/kg),		Não reverteu área de lesão
AQF (50, 100,150 e 200 mg/kg).	Etanol, v.o. 1 h antes do etanol	↓ Área de lesão
AC (12.5, 25, 50, 100, 150 e 200 mg/kg)		↓ Área de lesão

- Os extratos CE e ME mostraram atividade gastroprotetora da mucosa no modelo de úlcera aguda induzida por etanol absoluto.
- O extrato ME demonstrou clara atividade dose-dependente a partir de uma dose mais baixa (100mg/kg), sendo mais eficiente que a droga de referência, lansoprazol, na proteção da mucosa. Além disso, este extrato, por ser polar, é o que mais se assemelha ao do uso popular de *Abarema cochliacarpos*. Estes motivos justificaram sua escolha para continuação dos estudos.
- O ME foi particionado produzindo-se as frações acetato de etila (AF), *n*- butanólica (AC) e aquosa (AQF). Nos ensaios biológicos realizados com estas frações, no modelo de indução de úlcera por etanol absoluto, AF não apresentou atividade gastroprotetora, entretanto AC e AQF foram hábeis em reduzirem significativamente as lesões provocadas pelo etanol, demonstrando atividade gastroprotetora.

Atividade Antiulcerogênica Gástrica

Fração butanólica do extrato metanólico de *Abarema cochliacarpos*

Abarema cochliacarpos	Modelo, Rota e período administração	Parâmetros	Mecanismos de ação
AC (150 mg/kg)	Toxicidade aguda *in vivo* v.o.	Não apresentou efeitos tóxicos	
	Ligadura do piloro, intra-dudodenal Após ligadura.		Atividade anti-secretória
	Muco aderido, v.o. 1 h antes da ligadura		↑ Produção de muco
	Ácido acético 30%, subserosa 14 dias consecutivos	Cura de úlceras pré-estabelecidas	
		↓ Área de lesão	↑ COX-2
		↑ Proliferação Celular	↑ HSP-70
			↑ VEGF

- Não apresentou efeitos tóxicos no ensaio de toxicidade aguda *in vivo*;
- A ação anti-secretória participa da atividade gastroprotetora;
- O aumento da produção de muco está envolvido na atividade gastroprotetora de AC;
- Não foi observado nenhum parâmetro de toxicidade no modelo de úlcera crônica;
- AC foi capaz de promover cura de úlceras pré-estabelecidas. Isto foi caracterizado tanto macroscopicamente, pela redução da área de úlcera, quanto microscopicamente pela ocorrência de proliferação celular na base e margem da úlcera. A indução de fatores relacionados à cura corrobora estes resultados. A expressão de COX-2 foi imunolocalizada na região regenerada da mucosa e HSP-70 na região da mucosa ulcerada. VEGF foi detectado no tecido de granulação.

Atividade Antiinflamatória Intestinal Aguda e Crônica

Abarema cochliacarpos	Modelo	Rota, duração e período de administração	Parâmetros cólicos	Parâmetros bioquímicos	Mecanismo de ação
AC 100 mg/kg	TNBS, agudo 10mg/animal	Oral, 48, 24h, 1 hora antes e 24 horas depois do TNBS	Não reverteu: peso corporal, relação peso/comp. cólon, diarréia e adesões ↓ Lesões macroscópicas	↓ MPO ↓ COX-2 ↓ i-NOS -Não reverteu níveis de IL-10; - Não reverteu níveis de TNF-α	Não reverteu a ativação e p-JNK
AC 150 mg/kg			Não reverteu peso corporal, peso/comp. cólon e diarréia. ↓ Lesões macroscópicas ↓ Adesões	↓ MPO ↓ TNF-α ↓ COX-2 ↓ i-NOS -Não reverteu níveis de IL-10	↓ p-JNK
AC 150 mg/kg	TNBS, crônico 30 mg/animal	Oral,14 dias consecutivos após a indução por TNBS	Não reverteu peso do corpo; ↓ Diarréia ↓ Adesões ↓ Peso/ comp. cólon ↓ Lesões macroscópicas	↓ MPO ↓ TNF-α ↓ COX-2 ↓ i-NOS ↑ IL-10	↓ p-JNK

- No modelo agudo, AC foi hábil em reverter parâmetros cólicos e bioquímicos, principalmente na dose mais alta, 150 mg/kg, mostrando-se que esta fração possui substâncias ativas que possui efeitos anti-inflamatórios;

- Utilizando-se no modelo crônico, AC 150 foi capaz de reverter parâmetros cólicos (diarréia, adesões, e a relação peso/comprimento do cólon), como também a diminuição da atividade da MPO e a modificação dos níveis da citocina Th1, TNF-α, bem como a indução de *down-regualtion* de proteínas inflamatórias, COX-2 e i-NOS. Porém, apenas neste modelo AC 150 foi capaz de aumentar a produção da citocina antiinflamatória IL-10, sugerindo-se que o *shift* ↓TNF-α (Th1) ↑IL-10 (Th2) é um dos mecanismos antiinflamatórios promovidos por esta fração.

- Em ambos os modelos, AC foi hábil em inibir a ativação de JNK, na dose de 150 mg/kg.

VI Conclusões

Os extratos clorofórmico e metanólico de *Abarema cochliacarpos*, bem como frações aquosa e butanólica do extrato metanólico, apresentaram atividade antiulcerogênica em úlceras induzidas por etanol. A fração butanólica do extrato metanólico (AC150) também apresentou atividade gastroprotetora no modelo de úlcera crônica induzida por ácido acético. Os mecanismos de ação desta fração envolveram diminuição dos parâmetros secretórios de ácido, aumento da produção de muco e da expressão de COX-2, VEGF e HSP-70.

A fração butanólica da planta estudada também mostrou atividade antiinflamatória no modelo de colite ulcerativa induzida por TNBS. O mecanismo envolvido nesta ação parece estar relacionado à diminuição da atividade de MPO, *down-regulation* da expressão de COX-2 e i-NOS e diminuição da ativação de JNK no tecido cólico. Esta fração também foi hábil em diminuir os níveis de TNF-α, mas IL-10 só foi aumentada quando AC150 foi utilizada no modelo crônico.

VII Perspectivas

- Estudar outros mecanismos de ação envolvidos na atividade antiulcerogênica e antiinflamatória intestianl de *Abarema cochliacarpos*;

- Realizar estudos etnofarmacológicos de outras espécies medicinais da Caatinga dos Estados de Alagoas e Sergipe, visando também estudar sua atividade antiulcerogênica e antiinflamatória intestinal.

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