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ATIVIDADE ANTIULCEROGÊNICA DA DESIDROCROTONINA E DO ÓLEO ESSENCIAL OBTIDOS A PARTIR DAS CASCAS DE *Croton cajucara* Benth., UMA PLANTA DA FAMÍLIA Euphorbiaceae.

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Orientadora: **Profa. Dra. Alba R. M. Souza Brito**

Co-Orientador: **Prof. Dr. Domingos Sávio Nunes**

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"De tudo, ficaram três coisas:
A certeza de que estava sempre começando,
a certeza de que era preciso continuar e
a certeza de que seria interrompido antes de terminar.
Fazer da interrupção um caminho novo,
da queda um passo de dança,
do medo uma escada,
do sonho uma ponte,
da procura um encontro..."

Fernando Sabino

Ao meu pai Masao e minha mãe Kiyoe pelo eterno apoio e confiança.

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SUMÁRIO

RESUMO

Croton cajucara Benth. (sacaca) é uma espécie vegetal da região amazônica popularmente utilizada para o tratamento de distúrbios do trato digestivo. Foram investigadas a atividade antiulcerogênica e o mecanismo de ação dos constituintes majoritários obtidos a partir das cascas: o óleo essencial e a desidrocrotonina – DHC (uma sesquiterpenolactona presente em grande quantidade nas cascas). Através de modelos de úlceras agudas, induzidas por agentes como etanol, HCl/etanol, ligadura do piloro, indometacina e estresse, em ratos e camundongos, foi possível constatar que o óleo essencial e a DHC reduziram significativamente as lesões ulcerativas nos diversos modelos estudados. A partir destes resultados foram investigados os possíveis mecanismos antiulcerogênicos da DHC e do óleo essencial. A DHC não foi capaz de aumentar a produção de muco em células glandulares isoladas de estômago de ratos, e nem de apresentar atividade antioxidante em mitocôndrias isoladas de fígado de rato. Por outro lado, a DHC antagonizou, de modo não-competitivo, os receptores H_2 de histamina no átrio isolado de cobaia e M_1 no fundo de estômago isolado de rato; adicionalmente, apresentou um aumento na produção/síntese de PGE_2 pelas células da mucosa gástrica em ratos. A DHC não foi capaz de cicatrizar as úlceras pré-estabelecidas em ratos após 14 dias de tratamento. O óleo essencial alterou a acidez e o volume do suco gástrico quando administrado por via intraduodenal, o que demonstra uma ação sistêmica; aumentou a produção de muco pelas células glandulares do estômago de ratos e aumentou a produção/síntese de PGE_2 pelas células da mucosa gástrica em ratos. Em adição, o óleo essencial foi capaz de cicatrizar (32% de cura) as úlceras pré-estabelecidas em ratos, após 14 dias de tratamento, com a mesma potência da

cimetidina. Os resultados obtidos indicam que a utilização popular do infuso de sacaca, o qual contém DHC e óleo essencial, em distúrbios gastrointestinais, está perfeitamente justificado pela atividade de seus constituintes majoritários.

I - INTRODUÇÃO

Muitos trabalhos tem sido propostos na tentativa de elucidar a etiologia das úlceras. Atualmente, acredita-se que a úlcera gástrica dependa não somente da acidez gástrica e duodenal, mas também da presença de fatores pré-disponentes os quais atuariam, coletivamente, reduzindo a defesa das mucosas (Hirschowitz et al., 1995).

A úlcera, baseada em sua fisiopatologia, tem sido objeto de intensa pesquisa nas últimas décadas. Aproximadamente 10% da população ocidental e mais de 10% da asiática, independentemente do sexo e da classe social, é vítima desta patologia (Berstad & Berstad, 1993). Esta doença traz tremendos custos à sociedade (Jensen, 1984) e torna a vida do paciente extremamente difícil, além de ser letal em muitos casos (McIntosh et al., 1991; Petersen et al., 1995).

É conhecido que existem 5 fatores ambientais para o desenvolvimento da úlcera péptica: (a) a utilização cada vez mais freqüente de drogas antiinflamatórias não-esteroidais (DAINE), (b) a presença de *Helicobacter pylori* na mucosa do trato gastrointestinal (TGI), (c) o hábito de fumar, (d) o estresse ambiental e (e) os hábitos alimentares das populações (Lam, 1994).

Além disso, verificou-se que o fenômeno das migrações populacionais e o estresse a elas associado interferem com a incidência da úlcera (Sonnenberg, 1985; 1995) e que existe uma correlação importante entre o aumento de idade e a incidência de úlcera, para ambos os sexos (Ostensen et al., 1985). Outros fatores tais como a morte de parentes próximos e o aumento crescente da participação da mulher no mercado de trabalho vem sendo considerados também como fatores pré-disponentes de aumento das lesões gastrointestinais (Petersen et al., 1995). Tarnawski et al., (1995) citaram inúmeros fatores implicados na

etiologia das úlceras gastroduodenais, dentre os quais destacam-se aqueles genéticos, neurais, humorais, iatrogênicos (como a utilização de DAINE e esteroidais), emocionais e infecciosos, causados principalmente, pelo *Helicobacter pylori* (Taylor & Blaser, 1991).

Para Katsura et al., (1991) e Szabo et al., (1995) as úlceras pépticas são resultantes de um desequilíbrio entre os fatores defensivos da mucosa que representam forças de resistência ao suco gástrico como as secreções de muco e bicarbonato (HCO^{-3}) e aqueles agressivos, como por exemplo, a secreção de ácido, de pepsina e de fator intrínseco, os quais levam à sua lesão.

Desde que a secreção ácida foi descrita tornou-se evidente que, de alguma forma, a presença de ácido no estômago é nociva, além de principal responsável pela maioria das desordens que afetam este órgão, bem como do esôfago e duodeno (Peters & Richardson,1983; Wolfe & Sol, 1988; Sachs et al., 1994). Atualmente, entretanto, já se sabe que a secreção ácida e sua regulação, não podem ser considerados como os únicos fatores responsáveis pela úlcera péptica. O processo ulcerativo é complexo e exibe facetas múltiplas, as quais devem ser, uma a uma, analisadas. Alguns exemplos são:

- refluxo do conteúdo duodenal pode levar às úlceras gástricas. Apesar de resistir a um pH próximo de dois, as células epiteliais da mucosa gástrica não são resistentes aos ácidos biliares que rapidamente rompem a barreira gástrica mucoprotetora (Dayal & DeLellis, 1991; Sanioto, 1991; Wallace & Granger, 1996; Kutchai, 1996);
- esvaziamento gástrico acelerado pode levar às úlceras duodenais. Apesar de bastante resistente aos ácidos biliares, a mucosa duodenal não apresenta

resistência ao ácido gástrico, o qual não teve tempo de ser adequadamente neutralizado (Dayal & DeLellis, 1991; Sanioto, 1991; Kutchai, 1996);

- se a secreção de muco ou HCO_3^- for suprimida, a barreira da mucosa gástrica fica comprometida e os efeitos do ácido clorídrico e da pepsina sobre a superfície do estômago podem produzir úlceras (Baron et al., 1986; Curtis et al., 1995; Tarnawski et al., 1995; Kutchai, 1996; Wallace & Granger, 1996).
- agonistas α-adrenérgicos como a noradrenalina, além de produzirem vasoconstricção (impedindo a retirada do ácido coletado) e contração dos esfíncteres (o que retém o ácido no estômago), produzem ainda diminuição na secreção de HCO_3^- (Sanioto, 1991; Kutchai, 1996).

É possível verificar, assim, que apesar do importante papel da secreção ácida, a alteração de fatores protetores da mucosas, tais como produção de muco, HCO_3^- e prostaglandinas, também contribui para a instalação do processo ulceroso (Tarnawski et al., 1995).

1.1. Secreção Ácida Gástrica

O suco gástrico normal é uma mistura da secreção parietal (ácido e fator intrínseco) e secreções não parietais (muco, HCO_3^-, Na^+, K^+ e pepsinogênio) (Kutchai, 1996).

Os mecanismos moleculares da secreção de ácido clorídrico pela célula parietal iniciam-se através do estímulo de proteínas específicas de membrana por seus respectivos agonistas (acoplamento agonista–receptor), os quais são responsáveis por desencadear uma cascata de alterações bioquímicas intracelulares que irão, subseqüentemente, promover a secreção da célula

parietal (Wolfe & Sol, 1988; Sachs et al., 1994; Hirschowitz et al., 1995).

Três substâncias químicas endógenas constituem os principais agonistas responsáveis por estimular a célula parietal a secretar ácido clorídrico:

- Acetilcolina: atuando em receptores muscarínicos, provavelmente M_3, é liberada por neurônios eferentes vagais e estimula a secreção ácida da célula parietal pela elevação dos níveis intracelulares de Ca^{+2}. A excitabilidade produzida está associada a estímulos provenientes do olfato, visão, paladar ou mastigação e por neurônios locais da parede gástrica que são estimulados pela distensão das paredes do estômago (Hirschowitz et al., 1995; Kutchai, 1996);
- Gastrina: estimulando os receptores da colecistocinina-B (CCK-B) da célula parietal, induz o aumento da secreção ácida através do aumento dos níveis intracelulares de Ca^{+2}, assim como a acetilcolina. Acredita-se que a gastrina seja secretada pela presença de proteínas nos alimentos, Ca^{+2}, Mg^{+2}, Al^{+3}, além da estimulação vagal e da alcalinização do antro (Dockray et al., 1995; Konturek et al., 1995; Kutchai, 1996);
- Histamina: atuando em receptores H_2, é liberada através de um mecanismo parácrino por células "enterocromafins-like" (ECL), semelhantes aos mastócitos, existentes na lâmina própria do estômago, e dispostas em íntimo contato com as células parietais. A histamina promove aumento da secreção ácida por elevar os níveis intracelulares de AMPc (Sachs et al., 1994; Hirschowitz et al., 1995; Dockray et al., 1996; Sandor et al., 1996).

Os mecanismos celulares envolvidos com a secreção ácida iniciam-se com os estímulos proporcionados pelos agonistas acima citados, que atuam

sinergicamente conferindo uma elevada eficiência a este processo fisiológico.

Inicialmente, na célula parietal, ocorre o efluxo do íon Cl^- e com o intuito de assegurar a neutralidade elétrica no lúmen gástrico, o íon K^+ o acompanha por difusão. O fornecimento de K^+ à superfície extracelular é etapa imprescindível para a secreção ácida da célula parietal. Várias H^+/K^+ ATPases, localizadas em túbulos ou vesículas citoplasmáticas, após o estímulo, fundem-se com a membrana plasmática na superfície luminal das células parietais. Esta fusão aumenta a área de superfície canalicular pela formação de microvilos. As H^+/K^+ ATPases atuam como bombas de prótons na etapa final da secreção trocando o íons H^+ pelo K^+. Assim sendo, os íons H^+ e Cl^- formam uma solução extremamente ácida importante para as funções fisiológicas do estômago (Lind et al., 1983; Hirschowitz et al., 1995).

1.2. Mecanismos de Proteção da Mucosa Gástrica

Na proteção da mucosa gástrica e duodenal contra a agressão causada por ácido clorídrico, pepsina, bile, enzimas pancreáticas e outros fatores agressivos, vários mecanismos são considerados importantes. Entre eles podem ser destacados:

a) Muco

É um gel formador de um fino revestimento protetor sobre as células superficiais da mucosa com múltiplas funções. Na realidade o muco é composto de glicoproteínas que, em conjunto, recebem o nome de mucina, possuindo um aspecto viscoso. A atividade citoprotetora do muco se resume em proteger a

mucosa contra as forças mecânicas da digestão, retendo água e diminuindo a difusão de íons H^+ da luz para a membrana apical das células parietais, além de lubrificar a superfície gástrica (Forte, 1986). Por sua vez, o HCO_3^-, em altas concentrações, alcaliniza o muco que neutraliza o ácido luminal (Garner et al., 1984; Wallace & Granger, 1996).

Trier et al. (1987), Miller (1983) e Szabo (1987) demonstraram a importância da barreira de muco da mucosa gástrica através de experimentos de úlcera induzida por etanol, o qual romperia esta barreira e, deste modo, entraria em contato direto com a mucosa.

b) BICARBONATO

A proteção da mucosa estomacal não depende somente do controle da secreção ácida gástrica; há necessidade ainda de uma secreção adequada de muco e HCO_3^- pelas células mucosas cervicais e superficiais do epitélio (Wallace & Granger, 1996). Tanto a secreção de muco quanto aquela de HCO_3^- são estimuladas por diversos fatores e formam a chamada "barreira mucoprotetora" (Sanioto, 1991; Kutchai, 1996). O papel desta barreira na proteção da mucosa contra injúrias induzidas por ácido ou pepsina, tem sido objeto de intensa discussão (Wallace & Granger, 1996).

O HCO_3^- é secretado pelas células superficiais do estômago e pelas glândulas de Brünner do duodeno; permanece, em grande parte, abaixo ou na camada mucosa. Assim, a superfície mucosa fica em contato com o líquido que contém um alto pH em relação à luz do estômago que, em condições normais, irá neutralizar os íons H^+, enquanto difunde-se através da camada de muco,

estabelecendo-se um gradiente de pH entre a luz e as células epiteliais (Curtis et al., 1995; Wallace & Granger, 1996; Kutchai, 1996).

c) PROSTAGLANDINAS (PG'S)

Existem vários estudos sobre o papel das PG's exógenas sobre o estômago; entretanto, o modo pelo qual as PG's endógenas influenciam a fisiologia do estômago não está totalmente esclarecido.

Prostaglandinas compreendem uma família muito grande de ácidos graxos oxigenados de cadeia longa saturada (Miller, 1983). Aproximadamente 20 destas substâncias químicas tem sido identificadas em tecidos e fluídos corporais, e mais de uma centena destas tem sido sintetizadas como análogos ou derivados das PG's naturais. Altas concentrações podem ser encontradas no fluído seminal e na mucosa gastrointestinal.

Dentre os muitos fatores que podem contribuir para a atividade citoprotetora das PG's estão: a) estimulação da secreção de muco e HCO^{-3} (Garner et al., 1979; Garner et al., 1984; Eberhart & Dubois, 1995); b) manutenção do fluxo sangüíneo gástrico adequado durante exposição a algum tipo de irritante (Guth et al., 1984; Eberhart & Dubois, 1995); e c) inibição da liberação de mediador químico de mastócitos nas respostas inflamatórias (Hogaboam et al., 1993; Eberhart & Dubois, 1995).

As funções vasodilatadoras e protetoras das PG's na mucosa gástrica têm sido extensivamente documentadas e estudadas (Whittle & Vane, 1987; Gislason et al., 1995).

Ao nível molecular, as PG's do tipo E_2 inibem a adenilatociclase ativada

pela histamina nas células parietais (Major & Scholes, 1978; Eberhart & Dubois, 1995). Portanto, a PGE_2 inibe as respostas fisiológicas que seriam ocasionadas pela secreção de histamina, dentre elas, a mais importante, é a secreção ácida gástrica pelas células parietais.

Alguns estudos demonstraram, adicionalmente, que a síntese de PG's eleva-se significativamente quando o pH do lúmen gástrico torna-se ácido (Aly et al., 1985; Curtis et al., 1995) ou quando a secreção ácida é estimulada (Whittle & Vane, 1987). Além disso, existem evidências de que a síntese de PG's também eleva-se quando a mucosa gástrica permanece exposta a algum tipo de irritante (Robert et al., 1983).

A síntese de PG's tem sido sugerida ainda como moduladora frente a outros fatores como situações de estresse (Wallace & Cohen, 1984) e ativação de nervos colinérgicos aferentes, como por exemplo, utilizando altas doses de capsaicina (Goneda & Taché, 1993).

Portanto, as prostaglandinas, além de exercerem um importante papel na manutenção da integridade da mucosa gástrica, também são capazes de modular a secreção ácida gástrica (Soll, 1986; Curtis et al., 1995).

As PG's dos tipos E, F e I são encontradas nas mucosas gástrica e intestinal, sendo responsáveis pela secreção de muco e HCO_3^- e pelo aumento do fluxo sangüíneo da mucosa. As PG's da mucosa do duodeno parecem estimular ainda a secreção basal de HCO_3^- duodenal e sua resposta ao ácido luminal, além de propriedades citoprotetoras que auxiliam na regulação dos mecanismos de defesa da mucosa (Cho & Ogle, 1992; Eberhart & Dubois, 1995; Tabata et al., 1996).

Berglindh & Hansen (1984), utilizando culturas de células parietais isoladas de cães. observaram que a indometacina acentuava a resposta secretora das células parietais à histamina. Posteriormente, Waldum et al.,(1991) demonstraram que a utilização de indometacina induz danos à mucosa gástrica que levam à liberação de histamina pelas células ECL. Este dados, aliados à característica das DAINE de bloquear a síntese de PG's, são fatores importantes na caracterização dos fatores ulcerogênicos bem como definem a importância fisiológica na manutenção da integridade da mucosa estomacal (Gislason et al., 1995; Curtis et al., 1995)

Mas, os mecanismos citoprotetores pelos quais as PG's atuam sobre a mucosa gástrica, ainda permanecem pouco compreendidos (Curtis et al., 1995).

d) FLUXO SANGÜÍNEO

As mucosas gástrica e intestinal são supridas por capilares ramificados que atravessam a área glandular do estômago e do duodeno. Um grande plexo de artérias e veias, localizado na camada submucosa, regula o suprimento sangüíneo para as células epiteliais superficiais, o qual é importante na manutenção da integridade da mucosa (Morimoto et al., 1994; Tabata et al., 1996; Wallace & Granger, 1996; Kalia et al., 1997).

e) SOMATOSTATINA

Armazenada nas células D localizadas na mucosa do fundo e do antro gástrico, a somatostatina constitui-se em outro agente importante relacionado à proteção da mucosa, sendo responsável pela inibição das funções das células ECL e G (Sachs et al., 1994).

Desta forma, sugere-se que a regulação da secreção ácida pelas células endócrinas gástricas envolve um controle de retroalimentação positiva e negativa relacionada à tríade das células G, ECL e D, além da síntese de PG's na mucosa gástrica (Sachs et al.,.1994; Hirschowitz et al., 1995).

1.3. Terapêutica das Úlceras Pépticas

As drogas antiulcerogênicas são tão importantes do ponto de vista de utilização e, consequentemente, mercadológico que elas representaram, só no ano de 1992 nos Estados Unidos, um volume de vendas de US$ 4 bilhões (Alper, 1993).

As soluções terapêuticas, durante séculos. foram sempre dietas alimentares controladas, repouso absoluto, além da neutralização do conteúdo gástrico de ácido clorídrico utilizando antiácidos, ou em último caso, a intervenção cirúrgica (Jensen, 1984; Weir, 1988).

Nos anos 70 este panorama foi modificado com o trabalho de Black et al., (1972), onde os receptores H_2 da histamina foram definidos através da utilização de antagonistas seletivos do tipo da cimetidina e ranitidina. Estas drogas causaram um impacto tão grande, a nível mundial, que houve uma modificação significativa no panorama do tratamento de úlcera péptica (Kurata,1983). Algum tempo depois, devido ao papel desempenhado pelas PG's no estômago, principalmente as PG's E_2 e I_2, que inibem a secreção ácida gástrica e estimulam os fatores de proteção da mucosa como o muco e HCO_3^-, foram desenvolvidas as drogas denominadas citoprotetoras. O misoprostol, um análogo da PGE_1, foi a

droga desenvolvida e extensas revisões sobre a droga tem sido escritas (Monk & Clissold, 1987).

Mais tarde, foi a vez dos inibidores da bomba protônica ou H^+, K^+ ATPase, responsável pela secreção ácida gástrica. A substância padrão deste grupo de drogas foi o omeprazol, o qual é capaz de inibir a secreção ácida por inativação da bomba através da formação de ligações dissulfeto entre as moléculas reagentes do omeprazol com a enzima (Lindberg et al., 1987; Sachs et al., 1988).

Uma outra substância desenvolvida concomitantemente ao omeprazol foi a pirenzepina, um anticolinérgico muscarínico M_1 seletivo, que também contribuiu para o tratamento da úlcera péptica nesta década (Texter & Reylly, 1982).

Assim, a evolução do tratamento das dispepsias em geral foi satisfatória até o final da década de 80 quando antiácidos, anticolinérgicos M_1, anti-histamínicos H_2, citoprotetores e inibidores da bomba protônica, usados individualmente ou em associações, alternaram-se como soluções terapêuticas para a doença ulcerosa péptica. Após este tempo, nenhuma nova droga surgiu para fazer parte do arsenal terapêutico; entretanto, novos usos foram encontrados para velhas drogas como os compostos de bismuto (Baron et al., 1986) e a carbenoxolona obtida a partir de um extrato da *Glycyrrhiza glabra* ou alcaçuz (Barrowman & Pfeiffer, 1982).

Além disso, Hornick (1987) com um trabalho importante, levantou a possibilidade de que a úlcera péptica fosse o resultado de uma infecção bacteriana, induzida por *Helicobacter pylori* (Barbosa et al., 1988; Queiroz et al., 1988). Assim, o modo como a mucosa gástrica se protege dos danos a ela

impostos sugeriu mudanças na conduta terapêutica da doença ulcerosa, utilizando, por exemplo, colóides ou compostos de bismuto, assim como a antibioticoterapia (Talley & Ormand, 1989; Berstad & Berstad, 1993).

Como ainda não existe no mercado uma droga que produza 100% de remissão nas úlceras gastroduodenais (Alper, 1993) e por existir, só no Brasil, centenas de milhares de casos envolvendo este tipo de morbidade, o que significa um problema de saúde pública enorme, estudar substâncias com potencial atividade antiulcerogênica é, não só importante, como vital.

Dados recentes na literatura demonstram a grande variedade de substâncias químicas isoladas de plantas que apresentaram experimentalmente atividade antiulcerogênica (Lewis & Hanson, 1991), o que por si só, favorece a opção de se trabalhar com esta classe terapêutica em especial. Por outro lado, das 250 mil espécies vegetais existentes no planeta, 100 mil estão no Brasil, o que representa uma infinita fonte de recursos da biodiversidade a serem investigados.

1.4. Plantas Medicinais como Alternativas Terapêuticas

A recente "redescoberta" de plantas úteis do ponto de vista medicinal é decorrente, em parte, da crise na medicina ocidental – uma desvantagem imposta pelos dos altos custos alcançados pelas drogas produzidas sinteticamente e pelo limitado efeito em várias doenças crônicas. A Organização Mundial de Saúde estima que 80% dos países em desenvolvimento confiam na medicina tradicional e destes, 85% fazem uso das plantas ou de seus extratos como substâncias ativas (Sheldon et al., 1997). Quando se considera o uso de drogas derivadas de

planta o número aumenta ainda mais. Entre 1959 e 1973 as plantas ou seus extratos foram usados para a descoberta de mais de 40 prescrições de drogas; este número dobrou em 1980 e continua a aumentar, apesar do curto espaço de tempo (Milner Jr, 1997).

A pesquisa envolvendo plantas medicinais é complexa, cara e apresenta um caráter interdisciplinar. Estes aspectos são importantes quando o objetivo da pesquisa é o de encontrar substâncias potencialmente úteis para a terapêutica.

Vários problemas tem sido detectados nas diversas etapas envolvidas neste tipo de pesquisa e muito tem sido discutido sobre como solucioná-los.

Na década de 70 as preocupações estavam restritas às metodologias empregadas para a triagem farmacológica dos extratos e à necessidade de equipes multidisciplinares para realizar a seleção da espécie e o isolamento da substância farmacologicamente ativa (Farnsworth & Bingel, 1977; Malone, 1977).

Mais tarde, na década de 80, acreditou-se que a solução de todos os problemas envolvidos com a pesquisa de plantas medicinais passavam, necessariamente, pelo suporte oferecido pela etnofarmacologia/estudo da etnobotânica (Posey, 1986) aplicado às plantas medicinais (Zethelius & Balick, 1982; Malone, 1983; Elisabetsky & Posey, 1986; Elisabetsky & Gely, 1987; Elisabetsky & Moraes, 1988).

Recentemente, novas abordagens envolvendo técnicas de monitoramento químico da substância farmacologicamente ativa, tem sido propostas na tentativa de objetivar a pesquisa com plantas medicinais (McChesney, 1996), sem esquecer os papéis da etnofarmacologia (Elisabetsky & Wannmacher, 1993) e da fitoquímica (Nakanishi, 1989), tão discutidos em passado recente.

A farmacologia pré-clínica envolvendo plantas medicinais, do mesmo modo, apresenta inúmeros obstáculos, os quais dificultam e tornam pouco objetivo este tipo de trabalho. Estas dificuldades são tanto maiores se este tipo de trabalho for realizado em países em desenvolvimento, onde não há política de medicamentos definida; não existe proteção de direitos intelectuais e, por isto, o investimento em pesquisa por parte das indústrias farmacêuticas nacionais inexiste, o que significa um atraso tecnológico intransponível (Souza Brito, 1996); além disso, até bem pouco tempo, o Brasil não possuía uma legislação para os medicamentos fabricados utilizando plantas como matéria prima e vários problemas decorriam deste fato (Souza Brito, 1995 a e b).

A experiência na pesquisa farmacológica envolvendo plantas medicinais indica que, no presente, para selecionar uma dada espécie para estudo, não basta apenas haver uma indicação popular de uso medicinal, um botânico para identificar a espécie, um químico disponível para isolar e determinar a substância ativa, ou um farmacólogo experiente para realizar os ensaios. É necessário a conjunção de todos os profissionais, num trabalho multidisciplinar e interativo, na busca de substâncias farmacologicamente ativas com potencial terapêutico (Souza Brito, 1996; Souza Brito & Nunes, 1997).

Além disso, um ponto importante a ser analisado quando se decide pelo trabalho aplicado, com uma dada classe terapêutica, em particular, é a incidência da morbidade na população; este tipo de dado reflete o binômio importância em saúde pública e mercado local e mundial para a substância potencialmente útil na patologia (McIntosh et al., 1991; Petersen et al., 1995).

1.5. Espécie Utilizada

A espécie *Croton cajucara* Benth. pertencente à família Euphorbiaceae, vem sendo popularmente comercializada no "Ver o Peso", de Belém do Pará sob a denominação popular de sacaca. Esta espécie é conhecida e utilizada pelos indígenas da região amazônica e seu nome significa "feitiço" (Simões et al, 1979).

A sacaca é um arbusto grande que pode alcançar de 4 a 6 metros de altura com folhas pecioladas, biglandulosas, estipuladas, lanceoladas, longo-agudíssimo-acuminadas, perinervadas, verdes ou pardacentas, com até 14 cm de comprimento (figura 1). As flores são reunidas em inflorecências dispostas em racemos terminais de 6 a 9 cm de comprimento e os frutos são tri-loculares (Pio Corrêa, 1984). Esta planta é endêmica da região amazônica com centro de dispersão no Estado do Pará (Simões, 1979). Em Belém sua utilização está relacionada a problemas hepáticos tais como icterícia, hepatite, malária e diabetes. Além disso, existem também várias indicações na região amazônica do uso do chá das cascas e folhas para dores estomacais, gastrites e úlceras gástricas (Simões, et al., 1979; Di Stasi, 1989; Van den Berg, 1982) (figura 2).

Uma revisão bibliográfica do gênero *Croton* mostrou que de outra espécie, a C. *sublyratus*, foram isoladas diterpenolactonas denominadas de plaunol A, B, C, D e E (Kitazawa et al., 1980), muito semelhantes à DHC isolada de *Croton cajucara*, as quais foram patenteadas como drogas antiúlceras. O problema relativo à não utilização das substâncias descritas e patenteadas pelo grupo de Kitazawa e colaboradores, foi de que o rendimento obtido era muito baixo; para a obtenção de 1,5 - 4,5 g das substâncias ativas puras era necessário partir de 85 kg de material vegetal seco. Já na espécie em estudo, são obtidas

entre 5 e 6 g de DHC para cada quilo de cascas do caule de *C. cajucara*, o que caracteriza um rendimento satisfatório para uma subtância isolada e purificada. O óleo essencial obtidos de suas cascas também apresentou um bom rendimento (1%) e é formado por compostos sesquiterpênicos de constituição variada (Nunes et al., 1998).

Figura 1 - *Croton cajucara* em seu habitat.

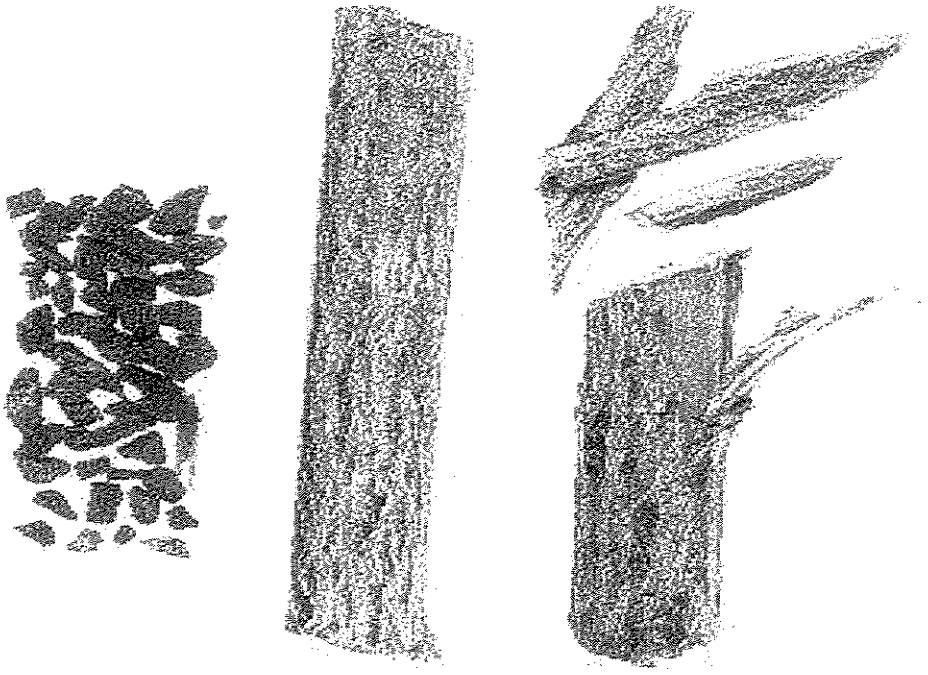

Figura 2- As cascas do caule *Croton cajucara*.

1.6. Modelos Experimentais

Existem diferentes modelos experimentais para o estudo de úlcera péptica em animais. Estes atuam por mecanismos que resumem, basicamente, o modo pelo qual as lesões gástricas aparecem na vida cotidiana do homem. Alguns modelos empregados serão rapidamente revisados:

- As úlceras causadas por agentes irritantes como o etanol e a solução de ácido clorídrico/etanol surgem devido à sua ação necrotizante na mucosa gástrica (Lewis & Hanson, 1991; Mizui & Doteuchi, 1983; Evans, 1996).
- A ligadura do piloro induz lesões gástricas por estímulo e acúmulo da secreção ácida no lúmen do estômago (Shay et al., 1945; Lewis & Hanson, 1991; Raffatullah et al., 1994).
- As DAINE, tais como a indometacina, causam lesões hemorrágicas na

mucosa gástrica decorrentes da inibição da síntese de prostaglandinas (PG's) e, consequentemente, diminuindo os mecanismos de citoproteção da mucosa gástrica mediados por estas substâncias. Além deste fator, podem também ser agregados o aumento da motilidade gástrica e a ativação de neutrófilos que conjuntamente atuam na indução da ulcerogênese (Morimoto et al., 1994; Takeuchi et al., 1994; Trevethick et al., 1995; Evans, 1996).

- Agentes parassimpatomiméticos como o betanecol atuam sensibilizando a mucosa gástrica através do estímulo da secreção ácida e pepsina facilitando, assim, a irritação gástrica causada pelas DAINE (Rainsford, 1987).
- Na úlcera induzida por estresse, as alterações no fluxo sangüíneo da mucosa gástrica e o estímulo para a secreção ácida, têm sido apontados como os principais fatores responsáveis pelo aparecimento das lesões (Yano et al., 1978; Raffatullah et al., 1990; Sato et al., 1995; Evans, 1996).

De um modo geral, os resultados obtidos nestes tipos de ensaios experimentais fornecem evidências, ainda que indiretas, de como drogas poderiam exercer seu efeito antiúlcera. Estes experimentos podem ser direcionados ainda para o desenvolvimento de estudos dos mecanismos de ação envolvidos com atividade detectada.

II - OBJETIVOS

Como a úlcera gástrica é uma patologia importante com alta incidência e, portanto, com um mercado significativo, foram objetivos deste trabalho investigar na espécie selecionada:

- a atividade antiulcerogênica aguda do óleo essencial e da desidrocrotonina (DHC) de *Croton cajucara* frente a diversos modelos de úlceras gástricas induzidas experimentalmente em ratos e camundongos.

- a atividade antiulcerogênica crônica ou atividade curativa do óleo essencial e da DHC em lesões gástricas previamente induzidas por ácido acético em ratos.

- os mecanismos de ação envolvidos com as ações antiulcerogênicas da substância isolada DHC e do óleo essencial em modelos experimentais diversos.

III – METODOLOGIA E RESULTADOS

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Natural Products and Medicinal Plant Research

Antiulcerogenic Activity of *trans*-Dehydrocrotonin from *Croton cajucara*

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Abstract: *trans*-Dehydrocrotonin (DHC), the major diterpene isolated from *Croton cajucara* Benth, was assayed for antiulcerogenic acitivity in four induced gastric ulcer models in the rat. At an oral dose of 100 mg/kg DHC showed a significant antiulcerogenic effect on ulcers induced by hypothermic restraint stress, ethanol, and pylorus ligature. No significant changes in indomethacin-induced gastric lesions or modifications in gastric parameters such as wall mucus, secretion rate, pH, and total acid content were found after DHC treatment. The acute toxicological effects of DHC were assessed in mice. The LD_{50} values were 876 mg/kg and 47.2 mg/kg for oral and intraperitoneal administrations, respectively. The cytotoxicity of DHC was also studied. A dose-dependent inhibition of cell viability was observed in V-79 fibroblast cell cultures with an IC_{50} of 240 μM. The high yields of DHC obtained from dried *C. cajucara* barks as well as its good antiulcerogenic activity and low toxicity support the pharmacological study of this compound as a potential new antiulcerogenic drug.

Key words: *Croton cajucara*, Euphorbiaceae, *trans*-dehydrocrotonin, antiulcerogenic activity, *in vivo* and *in vitro* toxicological effects.

Introduction

Bark and leaves of *Croton cajucara* Benth (Euphorbiaceae), an Amazonian medicinal plant commonly called "sacaca", are used traditionally to treat a wide range of gastrointestinal symptoms (1). Our ethnopharmacological field research on this species indicated an antiulcerogenic effect of the bark tea.

The nor-clerodane diterpene *trans*-dehydrocrotonin (DHC) is present in sacaca bark as the major secondary metabolite, suggesting an important role for this compound in the traditional preparation. Recent studies using classical models (2) have revealed that DHC exhibits some biological activities such as appetite-suppression (3). Clerodane derivatives with antiulcerogenic properties and structural similarity to DHC have been previously isolated from the Thai medicinal plant *C. sublyratus* (4).

We analyze here the antiulcerogenic activity of DHC in four different models of experimentally induced gastric ulcer in rats. Ethanol-, stress-, and indomethacin-induced gastric lesion models were used because they represent the most common causes of gastric ulcer in man. Moreover, we analyzed the effects of DHC on pylorus ligature which induces gastric lesions. The *in vivo* and *in vitro* acute toxicological effects of the compound were also determined.

Materials and Methods

Animals

Fasted male Wistar rats from the Central Animal House of the Universidade Estadual de Campinas (CEMIB/UNICAMP) weighing 150–250 g were used. Fasting was used prior to the ulcerogenic assays because standard drugs or DHC were always administered orally. Male Swiss albino mice weighing 30 ± 3 g were used to determine the acute toxicological effects of the compound. The animals received a certified Nuvilab CR-a® (Nuvital) diet and water ad libitum under standard conditions of 12 h dark-light period, humidity, and temperature.

Drugs

The following drugs were used: cimetidine (Tagamet® Smith-Kline), omeprazole (Losec®, Merrell Lepetit), Tween 80®, and indomethacin (Sigma Chemical Co, U.S.A.).

Isolation of trans*-dehydrocrotonin*

Barks of *C. cajucara* were collected in our experimental plantation in Benfica, near Belém, Pará, Brazil. A voucher specimen number 247 has been identified by Nelson A. Rosa and deposited in the herbarium of Museu Paraense Emílio Goeldi. Powdered plant material (20 kg dry bark) was extracted with hexane in a Soxhlet apparatus, and the crude crystals formed in a concentrated hexane solution were recovered after a few days (146 g). A pure compound (111 g) was obtained after repeated crystallizations from isopropanol, showing physico-chemical properties in perfect accordance with reported data for the structure of *trans*-dehydrocrotonin, as depicted in Figure 1 (2).

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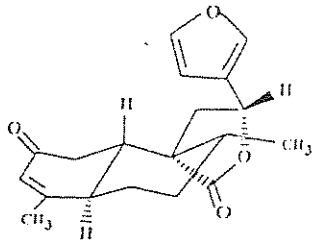

Fig. 1 Chemical structure of *trans*-dehydrocrotonin.

In vivo *toxicity*

Acute oral toxicity of DHC was tested on 12 h fasted male Swiss albino mice. Increasing doses of DHC were administered orally to groups of 10 animals for each dose level (125, 250, 500, 750, and 1000 mg/kg). Animals receiving the vehicle (12% Tween 80, 10 ml/kg) served as control. For the determination of intraperitoneal (*i.p.*) acute toxicity DHC was administered to groups of 10 animals at doses to 31.25, 62.5, 125, 250, and 500 mg/kg. The groups were observed at 0, 30, 60, 120, 180, and 240 min after DHC administration and then twice a day over the next 14 days and the number of survivors was recorded at the end of this period. The acute toxicological effect was inferred on the basis of mortality, expressed as LD_{50}. The oral or *i.p.* LD_{50} values were obtained using a software based on the method of Litchfield and Wilcoxon (5).

In vitro *toxicity*

The cytotoxic effect of DHC, expressed as cell viability (proliferate ability), was assessed on a permanent lung fibroblastic cell line derived from Chinese hamsters (V-79), commonly used for cytotoxicity studies (6). V-79 fibroblasts were grown as monolayers in Dulbecco's modified Eagle's MEM medium (DMEM). DMEM was supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. Cells were plated at 3×10^4 per well onto 24-well plates. The medium was removed 48 hours after cell seeding and replaced with medium containing DHC at concentrations ranging from 80 to 400 µM. The substance was dissolved first in methanol and then in DMEM. The final concentration of methanol in the test medium and control was 1%. Cells were exposed for 24 hours to test medium with or without DHC (control). Each drug concentration was tested in quadruplicate, with three replications. At the end of incubation the cell number in control and treated wells was estimated from total nucleic acid content according to Cingi et al. (6). Cells were washed twice with cold phosphate buffered saline solution (PBS) and the soluble nucleotide pool was extracted with cold ethanol. The cell monolayers were then dissolved in 0.5 M NaOH overnight at room temperature. Absorbance at 260 nm of the NaOH fraction was used as an index of cell number (6). Results are expressed as percent mean absorbance at 260 nm in treated wells compared to controls.

Acute gastric lesions

The antiulcerogenic activity of acute oral administration of DHC 100 mg/kg was assessed on 4 different experimentally induced gastric ulcer models.

(a) Shay ulcer: A total of 21 animals were randomly divided into three groups and fasted for 24 hours, with free access to water. Thirty minutes after oral administration of DHC (100 mg/kg), cimetidine (100 mg/kg) as positive control or vehicle, a 12% solution of Tween 80 (10 ml/kg), a pylorus ligature was performed as described by Shay et al. (7). The animals were killed 4 h later, the abdomen was opened and another ligature was placed around the esophagus close to the diaphragm. The stomach was removed and inspected externally, and its content drained into a graduated centrifuge tube and centrifuged at 2000 rpm for 10 min. The supernatant volume and pH were recorded. The total acid content of gastric secretion was also determined by titration to pH 7.0 with 0.05 N NaOH. Gastric lesions were evaluated by examining the inner gastric surface with a dissecting binocular microscope. Mucosal lesions were counted and the ulcerative index (UI) was determined according to the method of Sertié et al. (8).

(b) Indomethacin ulcer: A total of 21 animals were randomly divided into 3 groups and fasted for 24 hours, with free access to water prior to the experiment. Thirty minutes after oral administration of DHC (100 mg/kg), cimetidine (100 mg/kg), or 12% Tween 80 (10 ml/kg), 30 mg/kg of indomethacin were subcutaneously administered to unanesthetized rats of each group according to the method of Hayden et al. (9). Animals were killed 4 h later, the stomachs removed and opened and gastric lesions determined as described previously.

(c) Ethanol-induced ulcer: A total of 21 animals were randomly divided into 3 groups and fasted for 24 hours, with free access to water prior to the experiment. The ethanol-induced lesion assay was carried out according to the method of Morimoto et al. (10). One milliliter of 99.5% ethanol was orally administered to the animals which had been previously (1 h) treated with DHC (100 mg/kg), omeprazole (20 mg/kg), or 12% Tween 80 (10 ml/kg). One hour after ethanol administration the animals were killed, the stomachs were removed and opened and the ulcerative index was determined as described before.

(d) Restraint-hypothermic stress ulcer: A total of 19 animals were randomly divided into 3 groups and fasted for 48 hours, with free access to water prior to the experiment. Stress ulcer was induced as described by Levine (11). The gastric stress lesions were induced by placing the animals in a restraint cage at 4 °C. The rats were orally treated twice (18 h and 1.5 h) prior to the restraint-hypothermic-induced stress with DHC (100 mg/kg), cimetidine (100 mg/kg), and 12% Tween 80 (10 ml/kg), respectively. After 3 h the animals were killed, the stomachs removed and opened and the ulcerative index was determined.

Determination of gastric wall mucus

Gastric wall mucus was determined according to Rafatullah et al. (12). A total of 21 rats were randomly divided into 3 groups and fasted for 48 h, with free access to water. Each group was orally treated with DHC (100 mg/kg), carbenoloxone (200 mg/kg), and 12% Tween 80 (10 ml/kg), respectively. After 1.5 h, animals were submitted to stress as described previously. The animals were sacrificed, the stomach was removed and opened along the larger curvature. Glandular segments from the stomach were removed and weighed. Each segment was immersed for 2 h in 10 ml of 0.1% w/v Alcian blue dissolved in

0.16 M sucrose solution, buffered with 0.05 M sodium acetate, pH 5. Excess of dye was removed by washing the segments twice with 0.25 M sucrose solution during a period of 15 and 45 min, respectively. Mucus-dye complex was extracted by placing the gastric walls in 10 ml of 0.5 M magnesium chloride and shaking intermittently for 1 min at 30 min intervals for 2 h. Four milliliters of blue extract were mixed with an equal volume of diethyl ether and shaken vigorously for 2 min. The emulsion obtained was centrifuged for 10 min at 3600 rpm and the absorbance of the aqueous layer was recorded at 580 nm. Alcian blue extracted per gram of net glandular tissue was calculated.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical significance was determined by one-way analysis of variance followed by Duncan's test, with the level of significance set at $p < 0.05$.

Results

The LD_{50} values obtained by probit analysis for oral and intraperitoneal drug administration were 876 mg/kg ($r = 0.79 \pm 0.22$, $n = 10$, $p > 0.05$) and 47.2 mg/kg ($r = 0.978 \pm 0.07$, $n = 10$, $p < 0.05$), respectively.

The cytotoxicity assay showed a dose-dependent reduction of V-79 cell viability (Fig. 2). The IC_{50} value obtained mathematically from the concentration-response curves was 240 μM.

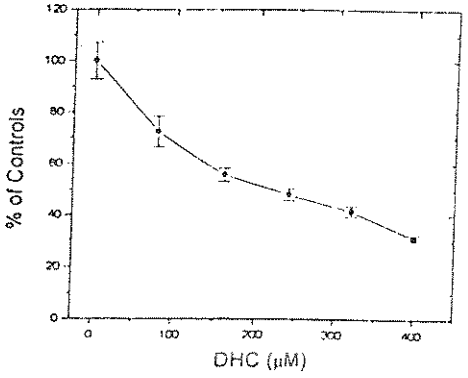

Fig. 2 Effect of 24 h DHC treatment on V-79 cell viability. Each point represents the mean ± SD of three experiments in quadruplicate. See Materials and Methods for details.

The effects of DHC on the four assayed methods of induced gastric lesions are shown in Figure 3. A single oral administration of DHC at the dose of 100 mg/kg inhibited the appearance of gastric lesions induced by hypothermic-restraint stress, ethanol, and pylorus ligature. The best inhibitory effect on the ulcerative index was observed in the hypothermic restraint-stress- and ethanol-induced gastric ulcer models (64.8 and 54 %, respectively). The effect of DHC (100 mg/kg) on the ulcerative index in the indomethacin-induced ulcer was not significant. In pylorus-ligated rats treated with DHC, the ulcerative index decreased with DHC pre-treatment. However, no significant modifications in gastric volume, gastric pH or total gastric acid content were observed (data not shown).

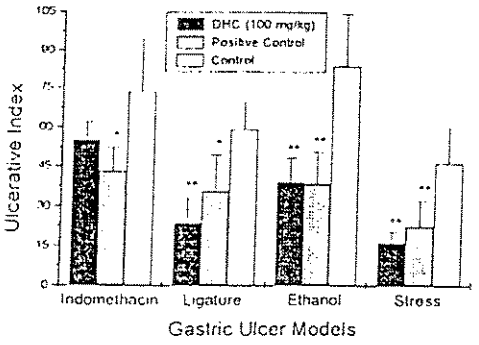

Fig. 3 Effects of DHC on gastric ulcer induced in rats by indomethacin (positive control: cimetidine), pylorus ligature (positive control: cimetidine), ethanol (positive control: omeprazole), and hypothermic-stress (positive control: cimetidine). Results are expressed as means ± SD. Asterisks indicate significant difference from corresponding control (ANOVA followed by Duncan's test where * $P < 0.05$; ** $P < 0.01$).

In hypothermic restraint stressed rats, the gastric wall mucus content was increased by carbenoxolone but remained unchanged after DHC treatment (data not shown).

Discussion

Our findings showed that acute oral administration of DHC to rats had toxicological effects comparable to those of drugs commonly used as therapeutics (13). The oral LD_{50} value is in good agreement with the theoretically expected LD_{50} calculated from the V-79 culture IC_{50} value using the Halle and Spielmann (13) relationship. Acute oral toxicity (LD_{50}) was induced by a dose eight times higher than the active antiulcer dose. The present results clearly indicate that the acute oral administration of DHC at a dose of 100 mg/kg had a significant antiulcer property, with no evident toxicological effects. The moderate toxicity observed in both *in vivo* and *in vitro* experiments support further study of this natural compound as a potential new drug.

In order to establish a general profile of the anitulcerogenic activity of DHC that would permit us to characterize its mechanism of action in a second step, we selected a fixed oral dose of 100 mg/kg. This dose was chosen because it is the same as our standard drug, cimetidine, thus allowing us to compare the potencies of the two compounds. The study was based on different experimental models of peptic ulcer disease which operate by distinct mechanisms of ulcerogenesis (14). Hypothermic-restraint stress ulcers have been widely used experimentally for the evaluation of anti-ulcer activity in rats because of data reproducibility (15). At the selected dose, DHC significantly protected the gastric mucosa against hypothermic-restraint stress-induced ulcers in rats as effectively as cimetidine (100 mg/kg). Disturbances of gastric mucosal microcirculation, alteration of gastric secretion, and abnormal motility have been considered to be the pathogenic mechanisms responsible for stress-induced gastric mucosal lesions and gastric mucus depletion (16). However, the most important factor in the genesis of stress ulcer is the increase in gastric acid secretion, which is often termed as the "aggressive factor" (17). The antisecretory activity of DHC as observed in our Shay rat model might be important in

protecting the gastric mucosa against stress-induced ulceration.

DHC also significantly protected the gastric mucosa against ethanol, a well known necrotizing agent. Ethanol-induced gastric mucosal lesions are caused by the direct action of ethanol on the mucosa and gastric acid participates little in the formation of this lesion (18). Moreover, it is well known that ethanol-induced ulcers are not inhibited by anti-secretory agents like cimeticiine, but are inhibited by agents which enhance mucosal defensive factors (19). In the present study, DHC and omeprazole showed inhibitory effects in this model. These results further indicate that DHC may enhance gastric mucosal defensive factors, such as mucus and prostaglandins.

Indomethacin is a potent prostaglandin biosynthesis inhibitor and there is mounting evidence that an increase of certain endogenous prostaglandins can enhance gastric mucosal resistance against ulcerogenic agents (20). DHC was unable to produce a significant reduction of the gastric mucosal damage induced by indomethacin.

We then examined the effects of DHC and carbenoxolone on gastric mucus amount, which is considered to be closely related to the mucosal defensive mechanism. Although this is only one more factor, it has been reported that the reduction of gastric mucus is a possible cause of the lesion formation induced by ethanol (21). The biochemistry and pharmacology of carbenoxolone have been extensively investigated, showing that, like many plants drugs, its efficacy is based on its ability to stimulate mucus synthesis (22), among other activities.

Since a reduced gastric wall mucus content is observed in animals submitted to stress, we studied the effects of DHC and carbenoxolone on mucus production by the stress-induced gastric lesions. While carbenoxolone produced a significant increase, DHC did not change the amount of mucus in the glandular portion of the stomach of rats submitted to hypothermic-restraint stress, when both were orally administered. Therefore, we presume that the antiulcerogenic effects of DHC on ethanol- and stress-induced ulcers do not involve an increase in mucus synthesis and/or an increase in mucus retention.

In conclusion, the oral administration of DHC had a significant antiulcer activity with no apparent toxicological effects. This anti-ulcerogenic effect was not related to an increase in mucus synthesis and/or mucus retention and was probably due to an increase in mucosal defensive mechanisms, such as prostaglandin production. New experiments are currently underway to determine the antiulcer mechanism of DHC.

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ANTIULCEROGENIC MECHANISMS OF DEHYDROCROTONIN, A DITERPENELACTONE OBTAINED FROM *Croton cajucara* BENTH.

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Abstract

The bark of *Croton cajucara* Benth. is used in Brazilian folk medicine as an infusion to treat gastrointestinal disorders. The aim of the present study was to assess the mechanisms involved in the antiulcerogenic activity of dehydrocrotonin (DHC), a diterpene isolated from *C. cajucara* bark. We studied the effects of DHC on pylorus ligature (Shay) in mice treated with the drug (100 mg/kg) by the intraduodenal route. DHC did not induce any alteration in gastric volume in Shay mice but modified the pH and total acid concentration of gastric juice. Incubation of gastric juice with DHC did not reduce gastric acidity compared to control. We also investigated the effects of DHC on the response to histamine of right atria isolated from guinea pigs and on the response to carbachol of stomach fundus strips from rats. The concentration-response curves for the chronotropic effect of histamine in guinea pigs right atria were shifted to the right, with a significant decrease in maximum response, in the presence of DHC. Similar results were obtained with DHC (30 μM) for the concentration-response curves to carbachol in the isolated rat stomach. The ability of DHC to increase PGE_2 release from rat stomach mucous cells was also studied. We observed that DHC induced a significant increase in PGE_2 production (60% compared to control). In addition, the effects of DHC on the healing of acetic acid-induced gastric ulcer in rats were evaluated 14 days after acid injection. Oral administration of DHC (100 mg/kg per day) for 14 consecutive days had no effect on gastric ulcer healing in rats. Thus, the protective effect of DHC on induced gastric lesions could be due to synergistic effects, e.g., an increase in PGE_2 release and non-competitive antagonism of H_2- receptors and of muscarinic receptors. Whereas the former result represents an increase in the protective factors, the latter one shows a decrease in the aggressive factors against the gastric mucosa.

Key Words: *Croton cajucara*, Euphorbiaceae, Dehydrocrotonin, cytoprotective effect, antiulcerogenic activity, anti-secretory effect.

Introduction

It is generally accepted that peptic ulcers are caused by a failure in the balance between gastric aggressive factors and mucosal defensive factors (1). Robert et al. (2) reported that prostaglandins (PG), even at non-secretory doses, prevented gastric necrosis produced by necrotizing agents such as ethanol, 0.6 N HCl, 0.2 N NaOH, 25% NaCl and boiling water, and named this unique property of PG "cytoprotection". Many cytoprotective antiulcer drugs which intensify the gastric defensive factors such as gastric mucosa blood flow (3), glycoprotein (4), and the barrier system in mucosal tissue (5), have been applied to the clinical treatment of peptic ulcers. Acid and pepsin components are the main "aggressive factors" and for several decades, the dogma "no acid, no ulcer" has dominated the view of peptic ulcer disease treatment (6) by the use of antacids, H_2-receptor antagonists and proton pump inhibitors.

The bark and leaves of *Croton cajucara* Benth. (Euphorbiaceae), an Amazonian medicinal plant called "sacaca", are commonly used as an infusion in the powdered or dried pill form to treat a wide range of gastrointestinal symptoms (7). Our research on the ethnopharmacological aspects of this species led us to determine the possible antiulcerogenic effect of the bark tea (8). The nor-clerodane diterpene *trans*-dehydrocrotonin (DHC) is present in the "sacaca" bark as the major secondary metabolite, indicating a possible important role for this compound in the traditional infusion preparation (9). Some previous studies have established the antinflammatory and hypoglycemic effects of DHC (10; 11). The similar antiulcerogenic clerodane representatives, plaunol A, B, C, D and E, were previously

isolated from the Thai medicinal plant *Croton sublyratus* (12).

We previously assayed DHC for antiulcerogenic activity on four induced gastric ulcer models in rats. DHC presented a strong antiulcerogenic activity on ethanol-, hypothermic restraint-stress- and pylorus ligature-induced gastric lesions. The inhibition of indomethacin-induced gastric lesions was weak and nonsignificant. In hypothermic restraint-stressed rats, the gastric wall mucus content was increased by carbenoxolone but remained unchanged after oral treatment with DHC (9). Our group has already speculated that the antiulcerogenic property of DHC should be due to an increase in mucosal defensive factors such as PG, mainly because DHC always showed an effect on gastric lesions induced by ethanol (9).

These previous results showed that DHC has antiulcerogenic activity as well as relatively low oral toxicity when administered for short periods of time. Thus, the data justify the pharmacological study of the mechanisms involved in the antiulcerogenic property of this compound as a potential new antiulcerogenic drug.

The objective of the present study was to assess the possible mechanisms involved in these pharmacological properties by investigating the effects of DHC on both the mechanism of gastric acid secretion and on the protection factors. We compared the antiulcer activity of DHC to that of cimetidine, an agent known to inhibit histamine-stimulated gastric acid secretion in the Shay model in mice, and we determined the curative effect of chronic oral administration of DHC and the effects of DHC on H_2- and on muscarinic receptors involved in gastric acid secretion using isolated preparations. We also investigated if the antiulcer effect of DHC could be mediated by prostaglandins as factors enhancing mucosal defense.

Methods

Animals

Male and female Wistar rats weighing 150-250 g and Swiss mice (25-35 g) from the Centro de Bioterismo of the Universidade Estadual de Campinas (UNICAMP) were used. Male guinea pigs (250-350g) from the Laboratório de Reprodução Animal of the Ministério da Agricultura were also used. The animals were fasted prior to all assays involving the stomach because standard drugs or DHC were always administered orally (by gavage -10 ml/kg) using a 12% solution of Tween 80 as vehicle. Animals received a certified Nuvilab CR-a® (Nuvital) diet and water *ad libitum* under standard conditions of 12 h dark-12 h light, humidity (55%) and temperature (22 ± 1°C). The protocols were approved by the UNICAMP Institutional Animal Care and Use Committee which follows the recommendations of the Canadian Council on Animal Care (13).

Drugs and Chemicals

The following drugs were used: cimetidine (SmithKline, Brazil), omeprazole (Merrell Lepetit, Brazil), Tween 80® (Synth, Brazil), carbamylocholine chloride, histamine dihydrochloride, propranolol hydrochloride, isoprenaline hydrochloride and indomethacin (Sigma Chem. Co, U.S.A.). The chemicals used in the nutritive solutions were all of analytical grade.

Isolation of trans-dehydrocrotonin

The bark of *C. cajucara* was collected from an experimental plantation in Benfica, near Belém, Pará, Brazil, and identified by Nelson A. Rosa. A voucher herbarium specimen has been deposited in the herbarium of Museu Paraense Emílio Goeldi under number 247. DHC was obtained as previously described by Souza Brito et al. (9). The physicochemical properties of the pure DHC were in perfect accordance with reported data (14) for the structure of trans-dehydrocrotonin depicted in Figure 1.

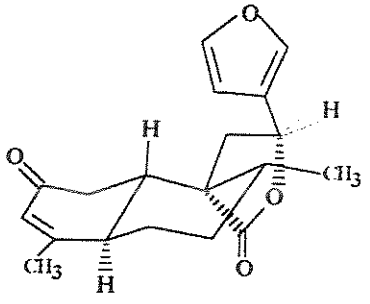


Figure 1. Chemical structure of *trans*-dehydrocrotonin.

Incubation of gastric juice

The abdomens of six male rats per group were opened under light ether anaesthesia, and the pylorus of each animal was ligated. The stomachs were removed 6 h later, the gastric juice obtained from each one was incubated with DHC (100 mg/kg) at 37 °C for 4 h in vitro, and the acid content was determined (15).

Determination of gastric secretion

solution (vehicle), indomethacin (20 mg/kg, s.c.) used as a positive control, and DHC (100 mg/kg, p.o.). The combination of DHC and indomethacin was assayed when DHC administration was followed 30 min. later by Indomethacin treatment. Indomethacin was dissolved in 5% sodium bicarbonate solution. We introduced sham animals to observe the PGE_2 level in animals without treatment. Thirty minutes after treatment, all the animals were killed by cervical dislocation and the abdomen was opened. A sample of the corpus (full thickness) was excised, weighed, and suspended in 1 ml of 10 mM sodium phosphate buffer, pH 7.4. The tissue was finely minced with a scissor and then incubated at 37°C for 20 min. PGE_2 in the buffer was measured by enzyme immunoassay (EIA; RPN222 - Armersham). The absorbance was read at 450 nm as previously described by Curtis et al. (21).

Statistical analysis

Results were expressed as mean ± SEM or SD. Statistical significance was determined by Student's t test when two groups were compared. One-way analysis of variance followed by Fisher's test or Dunnett's test with the level of significance set at $P < 0.05$ were used in the other experiments. All statistical analyses were performed using the Statistica 5.1 software (StatSoft, Inc.).

Results

The incubation of gastric juice with DHC did not produce any significant difference in gastric acidity when compared with the control group (data not shown).

The effects of DHC on biochemical parameters of the gastric juice obtained after submitting the animals to intraduodenal drug administration immediately after pylorus ligature are shown in Table 1. This table shows the antiulcer effect of the drug under study and of cimetidine used as a positive control. Both substances provoked a marked decrease in total gastric acid together with an increase in pH values. The volume of gastric juice was increased only in animals treated with cimetidine since the increase in this parameter observed in animals treated with DHC was not statistically significant. The fact that these effects were obtained only when DHC was administered intraduodenally seems to indicate that DHC has a systemic action.

The oral administration of cimetidine (100 mg/kg) for 14 consecutive days accelerated the healing of gastric ulcers in rats, producing a 32 % cure rate compared with the control group. However, DHC at the same dose, showed no significant effects (6 %) on the ulcer index in rats (Table 2).

The histamine H_2-receptor antagonist property of the test compound was determined by the inhibition of the chronotropic effect of histamine in isolated guinea pig atria. Spontaneously beating atrial basal rate was decreased in the presence of 30 μM DHC. Concentration-response curves to histamine in the presence of DHC were shifted to the right with decreases in the histamine pD_2 value (Table 3). The doses of 10 and 30 μM DHC also induced a decrease in the maximum response to histamine (Figure 2A). In order to verify the specificity of this DHC effect, concentration-response curves to isoprenaline in the presence of the highest DHC concentration were also obtained. Although, a decrease in the maximum response was observed, the isoprenaline pD_2 value was not altered in the presence of DHC (Table 3, Figure 2B).

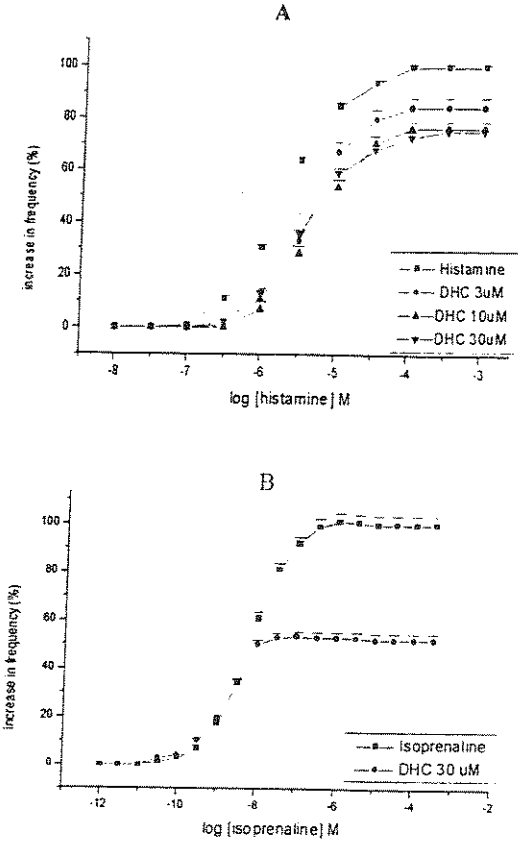


Figure 2. Cumulative dose-response curves to histamine (A) and isoprenaline (B) in the absence and presence of different concentration of DHC. Each point is the mean for 6-8 experiments.

DHC at the doses of 3 and 30 μM also shifted to the right the cumulative concentration-response curves to carbachol in rat fundus strips (Figure 3), with a decrease in the

A total of 19 mice were randomly divided into three groups. Animals were fasted for 24 hours with free access to water. The assay was performed by the method of Shay, with some modifications (16). The pylorus was ligated immediately after the intraduodenal administration of DHC (100 mg/kg), cimetidine (100 mg/kg) as positive control, or vehicle (10 ml/kg). The animals were killed 3 hours later and the abdomen was opened and another ligature was placed around the oesophagus, close to just below the diaphragm. The stomach was removed and internally inspected, and its content was measured and drained into a graduated tube which was centrifuged at 2000 rpm for 10 min. The pH was recorded with a digital pHmeter (PA 200, Marconi S.A. Brazil) and the total acid content of gastric secretion was also determined by titration to pH 7.0 with 0.01 N NaOH using a digital burette (E.M., Hirschmann Technicolor, Germany).

Acetic acid-induced gastric ulcers

The experiments were performed according to the method of Takagi et al. (17). Male Wistar rats (200-250 g) were anaesthetised with ether and the abdomen was incised. The anterior wall of the stomach was exposed and 0.05 ml of 30% acetic acid (v/v) was injected into the submucosal layer at the junction of the fundus with the antrum, about 1 cm proximal to the pylorus. DHC (100 mg/kg), cimetidine (100 mg/kg) or vehicle (10 ml/kg) was administered orally for 14 consecutive days, once a day, beginning 2 days after surgery. On the day following the last drug administration, the animals were sacrificed. The stomach was removed, fixed with formalin and incised along the greater curvature. Subsequently the ulcer area (mm^2) and the curative ratio (%) were measured as described by Takagi et al. (17).

Effect of DHC on guinea-pig isolated atrial sensitivity to histamine and isoprenaline

This experiment was conducted as previously described by Krielaart et al. (18). Male guinea pigs were killed by cervical dislocation. The heart was quickly dissected and the right atrium was isolated and mounted under 0.5 g of resting tension in a water jacketed organ bath containing Krebs-Henseleit solution of the following composition (mmol/l): NaCl, 115.0; KCl, 4.6; $CaCl_2.2H_2O$, 2.5; KH_2PO_4, 1.2; $MgSO_4.7H_2O$, 2.5; $NaHCO_3$, 25; glucose, 11.0, gassed with 95% O_2 - 5% CO_2 at 36.5 ± 0.1°C. The increase in heart rate (bpm) induced by histamine in a cumulative dose schedule was recorded with an isometric transducer (Narco Bio-System) coupled to a polygraph (Narco Bio-System). The β-adrenoceptors were blocked with 1 μM propranolol previously added to the Krebs-Henseleit solution. After obtaining the cumulative concentration-response curve to histamine, the preparations were washed for 45 min with 4 changes of bathing solution. The atria were pre-equilibrated with one of 3 different concentrations (3, 10 and 30 μM) of DHC for 30 min prior to the determination of another concentration-response curve for the same agonist. The same procedure was used when the agonist was isoprenaline. In this last experiment the cumulative concentration-response curves for isoprenaline were obtained in the presence of 1 μM cimetidine also added to the nutritive solution.

Muscarinic-receptor antagonism in rat isolated stomach fundus

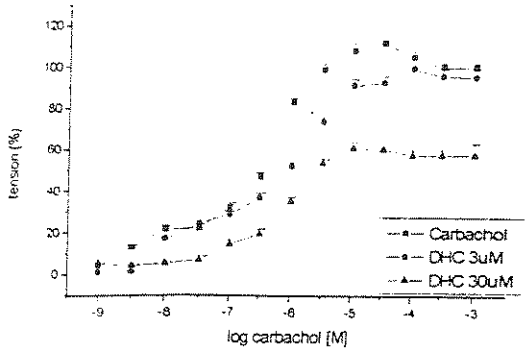
Male and female albino Wistar rats were deprived of food 8 h prior to the experiment as previously described by Korolkiewicz et al. (19). Animals were killed by cervical dislocation. The abdomen was opened with a midline incision, the stomach excised, the fundus dissected out and longitudinal strips were cut off according to the method described by Vane (20). The strips were placed into the Krebs-Henseleit solution as described for the atrial experiment. One end of the strip was attached to a fixed support and the other one, to a lever connected to an isometric transducer coupled to a polygraph (Narco Bio-System). The contractions induced by carbamylcholine chloride (carbachol) were measured as the tension developed by the tissue. The H_2 histaminergic receptor was blocked with 1 μM cimetidine present in the Krebs-Henseleit solution. Tissues were allowed to equilibrate for 60 min before the beginning of the experiment. The nutritive solution was changed every 10 min, except for the time of contact with DHC, which lasted up to 30 min. Cumulative concentration-response curves for carbachol in the absence and presence of different concentration (3 and 30 μM) of DHC were obtained.

Prostaglandin synthesis determination

All rats were deprived of food for 24 h prior to the experiment and all experiments were performed between 09:00 and 11:00 a.m. Groups consisting of at least 5 rats received one of the following solutions: 12 % Tween 80

maximum effect of the agonist in the presence of 30 µM DHC (Table 4).

Figure 3. Cumulative dose-response curves to carbachol in



the absence and presence of different concentration of DHC. Each point is the mean for 3-9 experiments.

The data obtained for detection of PGE_2 production using EIA by gastric tissue are shown in Figure 4. The treatment with DHC induced an increase on PGE_2 production which was significantly higher than the basal and control levels. The pre-treatment of these animals with indomethacin, inhibitor of cyclooxigenase enzyme, was efficient to inhibit this increase on PGE_2 production induced by DHC. These results show that DHC causes increase in PGE_2 production by activation of cyclooxigenase enzyme.

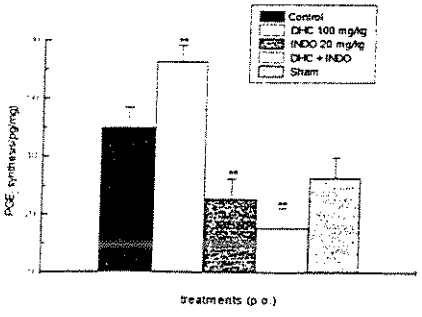


Figure 4: Effects of oral administration of DHC and indomethacin (INDO) on gastric PGE_2 production in rats. Each group is the mean ± SEM for 5-8 animals. ANOVA: $F_{(4,17)} = 13.0$, $P < 0.05$, followed by Fisher's test, ** $P < 0.001$.

Discussion

We have previously demonstrated the antiulcerogenic properties of trans-dehydrocrotonin isolated from *Croton cajucara* bark. DHC inhibited the gastric lesions induced in rats by ethanol, hypothermic-restraint stress and pylorus ligature, but not the ulcers induced by indomethacin (9).

In the present study we analysed the possible mechanisms involved in the antiulcerogenic activity of DHC. The incubation of gastric juice from control rats with DHC ruled out the hypothesis that DHC exhibits a direct neutralization effect (15). Another possibility previously reported would be a local action of the compound. The present results have shown that both DHC and cimetidine administered by the intraduodenal route provoke a marked reduction in total gastric juice acidity. An increase of total gastric juice volume was observed just with cimetidine although this parameter tended to increase in animals treated with DHC. We previously reported that DHC (p.o.) decreases the ulcerative index in Shay animals but does not alter the gastric juice parameters (9). So, these results confirm that both drugs exhibit a systemic action rather than a local effect.

It is well known that antisecretory drugs such as H_2 and/or muscarinic antagonist and proton pump inhibitors blocked the ulcerative lesions in the Shay model (22). We carried out a series of functional studies in which we analysed the response to different agonists using isolated preparations. We have decided to quantitate the effects of H_2-antagonists using spontaneously beating right atria from guinea pigs as a model for the H_2-receptor-mediated responses to histamine. A reason to choose the atrium model is that the system is hardly desensitized during the experiment and no time dependency of the agonist response was observed over periods of up to 300 min (18).

Our data have shown that in spontaneously beating right atria from guinea pigs, dose-response curves to histamine are shifted to the right in the presence of DHC, suggesting that DHC may act as an H_2-histamine receptors antagonist. The decrease in the maximum response to histamine induced by DHC also suggests that DHC may act as a non-competitive antagonist of H_2-receptors (23). On the other hand, it has been previously reported that the main drawback of all H_2-antagonists is that they cause a suppression of the maximally attainable biological effect at high concentrations (24; 25; 26). Kricelart et al (18) showed that curves for histamine obtained in the presence of antagonists such as cimetidine, ranitidine or famotine show a concentration-dependent decrease in the maximally inducible frequency.

Table 1. Effects of DHC on the biochemical parameters of gastric juice obtained from pylorus-ligated mice.

Treatment (p.o.)	Dose (mg/kg)	n	PH	Gastric Juice Volume (ml)	Total Gastric Acid (mEq/hour)
Control	-	6	3.66 ± 0.52	0.36 ± 0.03	6.02 ± 1.58
Cimetidine	100	6	6.13 ± 0.73**	0.65 ± 0.23*	1.87 ± 0.62**
DHC	100	7	6.04 ± 0.76**	0.56 ± 0.22	2.21 ± 1.24**

Expressed as mean ± SD. Fisher's test, * P< 0.05 ** P< 0.001.

The combination of rightward displacement of concentration-response curves with depression of the maximal effect could be explained by impairment of the intracellular stimulus-transfer process by the antagonist (27). The decrease in the maximum effect observed in the concentration-response curves to histamine obtained in the presence of DHC, was not abolished by isoprenaline as opposed to what occurs when the antagonists are those cited above (18). Moreover, since DHC also affected the basal rate of guinea pig atria, our data seem to indicate that these effects could be probably due to impairment of the second messenger system or calcium-entry blockade (28). On the other hand, dose-response curves for histamine were displaced to the right in a dose-dependent manner whereas pD_2 values of isoprenaline were not affected by the higher concentration of DHC. Thus, the effect of DHC on the response to histamine seems to include an antagonism at the receptor level.

We obtained some preliminary data on the effects of DHC in the isolated rat stomach fundus response to carbachol. Concentration-response curves to carbachol were shifted to the right together with a decrease in maximum response caused by the highest dose of DHC. These data provide additional support for our hypothesis that the main effect of DHC is on the intracellular mechanisms that follows receptor activation rather than at the receptor level.

The role played by H_2 or muscarinic receptor stimulating gastric acid secretion is well documented (29). The antagonism of H_2-receptor not only blocked histamine-induced acid secretion, but also gastrin-induced acid secretion and blocked much, but not all, of the vagally-mediated acid secretion (30).

Our functional studies revealed that there is a similar nonspecific pattern of the DHC effect on the response of different tissues to agonists of the gastric acid secretion. Although our data are not sufficient to speculate about the kind of interactions that may occur, there is no doubt that DHC interferes with gastric acid secretion since all the responses analysed were related to gastric acid secretion.

Among the many factors that may contribute to the protective actions of PG in the stomach are the stimulation of phospholipid, mucus and bicarbonate secretion, maintenance of gastric blood flow during exposure to an irritant, and inhibition of inflammatory mediator release from mast cells and of free radicals production (31). We previously reported that DHC did not increase mucus production (9). Still looking for other possible mechanisms that increase the mucosal protective factors, we investigated the effect of DHC on PGE_2 production. Our data demonstrated that DHC increases PGE_2 production, an effect which is completely abolished by pre-treatment with indomethacin. Therefore, DHC has an antiulcerogenic effect by increasing gastric juice volume with a low acidity plus enhancing PGE_2 synthesis. These two effects are observed when DHC is administered before lesion induction, which means that the compound has a preventive anti-ulcerogenic effect. However, we demonstrated that DHC has no healing effect on already installed lesions.

In conclusion, these results, taken together, suggest that DHC has an excellent preventive effect on gastric ulcers, mainly by suppressing acid secretion through a non-competitive antagonism with receptors involved in gastric acid secretion and by protecting the gastric mucosa by an increase of PGE_2. The curative effect of DHC on acetic acid-induced gastric ulcers was not observed under our experimental conditions. Further studies will be carried out to determine whether the latter data can be confirmed histologically.

Table 2. Effect of DHC on the healing process of ulcers produced by the injection of 30% acetic acid solution into the rat stomach. The ulceration was observed on the 14th days after surgery.

Treatment (p.o)	N	Dose (mg/kg)	Lesion area (mm^2)	Curative ratio %
Control	9	-	20.2 ± 1.55	-
Cimetidine	8	100	13.7 ± 1.87 *	32.2
DHC	7	100	19.6 ± 4.27	6.27

Expressed as mean ± SEM. Fisher's test, * P< 0.05.

Table 3: pD_2 values, basal rate (BR) and maximum response (MR) to Histamine and Isoprenaline in the absence and presence of DHC in isolated guinea pig atrium.

Agonist	Histamine[a]				Isoprenaline[b]	
Antagonist-DHC(μM)	0	3	10	30	0	30
pD_2	5.77 ± 0.04	5.34 ± 0.06**	5.33 ± 0.05**	5.47 ± 0.06**	8.17 ± 0.11	8.27 ± 0.05
BR (bpm)	192.9 ± 11.5	161.7 ± 13.5	168.0 ± 13.9	84.0 ± 10.3**	204.6 ± 5.1	133.3 ± 3.33*
MR (bpm)	141.4 ± 2.61	120.0 ± 11.6	108.0 ± 13.9*	106.7 ± 7.6*	173.0 ± 7.9	90.0 ± 6.83*

[a] Expressed as mean ± SEM. ANOVA: $F_{(3;20)}$ = 16.1(pD_2); 10.7(BR); 3.36 (MR); P< 0.05, followed by Dunnett's test, * P< 0.05 ** P< 0.001. [b] Expressed as mean ± SEM. Student T test * P< 0.05.

Table 4: pD_2 Values and Maximum Response (MR) to Carbachol and DHC in isolated rat gastric fundus.

Agonist	Carbachol		
Antagonist+ DHC (μM)	0	3	30
pD_2	6.39 ± 0.16	6.10 ± 0.11	5.51 ± 0.39*
MR (g)	2.15 ± 0.18	1.84± 1.58	1.15 ± 0.37*

Expressed as mean ± SEM. ANOVA: $F_{(2,17)}$ =6.68(pD_2) and 2.62(MR) for P< 0.05, followed by Fisher's test. *P< 0.05.

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Dear Editor,

We are sending the manuscript of our work "**ANTIULCEROGENIC EFFECTS AND SUBCHRONIC TOXICITY OF trans-DEHYDRO-CROTONIN OBTAINED FROM *Croton cajucara***" to be considered for publication in Pharmacology & Toxicology.

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Sincerely yours,

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ANTIULCEROGENIC EFFECTS AND SUBCHRONIC TOXICITY OF trans-DEHYDROCROTONIN OBTAINED FROM *Croton cajucara*

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Abstract

The anti-ulcerogenic activity of dehydrocrotonin, a nor clerodane diterpene isolated from *Croton cajucara* Benth (Euphorbiaceae) and its subchronic (35 days) toxicological effects were studied in mice and rats, respectively. In the HCl/ethanol-induced gastric ulcer model in mice, at oral doses of 100 and 200 mg/kg, trans-dehydrocrotonin (DHC) significantly reduced ($p < 0.001$) the formation of gastric lesions compared to the controls. Furthermore, ulcers induced by hypothermic restraint stress in mice were also reduced at an oral dose of 100 mg/kg ($p < 0.001$) as well as cimetidine used as a positive control at the same dose. However, DHC was unable to remove free radicals in rat liver mitochondrias. In subchronic toxicity experiment, rats were orally treated with DHC for 5 weeks at doses of 25, 50 and 100 mg/kg. At the end of treatment no differences were observed in body weight gain, water or food intake, urine/excrement output or haematological and urinary parameters. Nevertheless, a significant dose-dependent increase in liver weight was observed in both male and female rats, whereas a significant reduction in plasma alkaline phosphatase and cholesterol levels and an increase in gamma glutamyl transpeptidase were observed only at high doses (100 mg/kg) in female rats. Subchronic administration of DHC also induced histopathological alterations of the liver noted as turbid tumefaction, microvacuolate degeneration and nuclear alterations. Despite the observed anti-ulcerogenic activity, our results suggest that the long-term use of DHC induces liver damage.

Keywords: *Croton cajucara*; Dehydrocrotonin; anti-ulcerogenic activity; subchronic toxicity; nor clerodane diterpene.

Introduction

Croton cajucara Benth. (Euphorbiaceae) is a well-known medicinal plant used in Amazonian folk medicine to treat several illnesses such as diarrhoea, diabetes and liver inflammation, and to control high cholesterol levels (1, 2, 3). Bark and leaves from *C. cajucara* are commonly used as infusion in the powdered or dried pill form (3).

Several compounds occur in *Croton* species including essential oils (4) and different diterpenes (5, 6). Trans-dehydrocrotonin (DHC) is a 19-nor-clerodane diterpene isolated from *C. cajucara* bark. In previous studies DHC was found to be an insect growth inhibiting, anti-inflammatory and anti-nociceptive compound (3). Our ethnopharmacological field research on this species led us to determine the possible antiulcerogenic effect of the bark tea (7).

We previously assayed DHC for antiulcerogenic activity on four induced gastric ulcer models in rats. DHC presented a strong antiulcerogenic activity on hypothermic-restraint stress-, ethanol- and pylorus ligature-induced lesions. The inhibition of indomethacin-induced gastric ulcers was weak and non significant. We also observed that in hypothermic-restraint stressed rats, the gastric wall mucus content was increased by carbenoxolone but remained unchanged after oral treatment with DHC (8).

Considering these data, in the present study we analysed the antiulcerogenic effect of DHC in other ulcerogenic models and the possible toxic effects of its prolonged use. First, we investigated the effects of DHC on gastric lesions induced by another irritant, 0.3 M HCl / 60% ethanol, and by hypothermic restraint stress, both in mice. In the former model, we studied whether the effect of DHC is dose-dependent and compared it with that of lanzoprazole used as a positive control. In the latter model, the antiulcerogenic effect of DHC was compared with that of cimetidine, both used at the same dose and by the same route. Second, we investigated any possible effect of DHC in removing free radicals in rat liver mitochondria. It is well known that antioxidants can prevent some gastric lesions such as ethanol-induced gastric injury (9). We observed that DHC presented a low acute toxicity (DL_{50} = 876 mg/kg) when administered by the oral route (8). In view of this finding we also studied the subchronic (35 days) oral toxicity of DHC in rats to analyse the effects of its long-term use.

Material and Methods

Plant material

The bark of *Croton cajucara* was collected in Belém, state of Pará, Brazil, and identified by Nelson A. Rosa. A voucher herbarium specimen has been deposited at the Museu Paraense Emilio Goeldi under the number 247. DHC was obtained as previously described by Souza Brito et al. (8). Powdered dry bark was extracted with hexane in a Soxhlet apparatus and the crude crystals formed were recovered in a concentrated hexane solution. After repeated crystallization using isopropanol, pure DHC with its characteristic physicochemical properties was obtained (2). The yield in terms of dry starting material was 0.55 %.

Animals

Male and female Wistar rats from the Central Animal House of the Universidade Estadual de Campinas (UNICAMP) were used to evaluate the subchronic toxicological effects of DHC. Male Swiss albino mice weighing 30 ± 3.0 g were used for the anti-ulcerogenic activity assays. The animals were fed a certified Nuvilab CR-a® (Nuvital) diet with free access to water under standard conditions of lighting(12 h dark-light period), humidity (60 ± 10%) and temperature (21.5 ± 1.0 °C). The protocols were approved by the Unicamp Institutional Animal Care and Use Committee that follows the recommendations of the Canadian Council on Animal Care (10).

Anti-ulcerogenic activity

(a) HCl/ethanol-induced ulcer: The anti-ulcerogenic activity of DHC in HCl/ethanol-induced gastric ulcer was assessed in mice as described by Mizui and Doteuchi (11). Mice were divided into groups of 7-8 animals and fasted 24 h prior to receiving an oral dose of the vehicle, 12% Tween 80 (10 ml/kg), lansoprazole (20 mg/kg) or DHC (50, 100 and 200 mg/kg). After 50 min all groups were orally treated with 0.2 ml of a 0.3 M HCl/60% ethanol solution (HCl/ethanol) for gastric ulcer induction. Animals were sacrificed with ether 1h after the administration of HCl/ethanol, and the stomachs were excised and inflated by injection of saline (2 ml). The stomachs were fixed in 5% formalin for 30 min and opened along the greater curvature. Gastric damage visible to the naked eye was observed in the gastric mucosa as elongated black-red lines (1-10 mm long and 0.5-1.5 mm wide) parallel to the long axis of the stomach similar to the HCl/ethanol-induced lesions in rats. The extension of the lesions was measured, and the lesion index was expressed as the sum of all lesions. Results are expressed as an ulcerative index (UI) as described by Sertié et al. (12).

(b) Hypothermic stress-induced ulcer: The anti-ulcerogenic activity of DHC in the hypothermic stress-induced gastric ulcer model was assessed in mice according to Levine (13). Mice were divided into groups of 7 animals. After 24 h of starvation, the animals received an oral administration of DHC (100 mg/kg), cimetidine (100 mg/kg) or 12% Tween 80 (10 ml/kg). One hour after treatment, ulceration was induced by immobilizing the animals inside a closed cylindrical cage maintained at 4°C. After 3 h the animals were sacrificed with ether, and the stomachs removed and examined for ulcers as described above.

Isolated rat liver mitochondria

Liver mitochondria were obtained from 12 h fasted female Wistar rats weighing 200-250 g as previously described (14). The mitochondria pellet was resuspend in isosmotic buffer containing 120 mM KCl and 20 mM HEPES, pH 7.2, to give a final protein concentration of 80-100 mg. The final protein concentration was determined by the modified biuret method with the addition of cholate (15). Lipid peroxidation of liver mitochondria was determined by the formation of thiobarbituric acid reactive substances (TBARS) as described by Nepomuceno et al. (16). The DHC effect on TBARS formation was assessed at 100, 200 and 300 µM. Tubes containing 1 ml of reaction medium (120 mM KCl, 5 mM succinate, 2 µM rotenone and 20 mM HEPES, pH 7.4), 1 mg of protein (mitochondria suspension), 1% of methanol and 50 µM of $FeSO_4$ were incubated at 37 °C for 20 min in the absence (control) or presence of DHC, at the selected concentrations. DHC was dissolved in methanol and then added to the reaction medium to reach a final concentration of 1% methanol. After this incubation time, 0.1 ml of 50 µM butylhydroxytoluene was added to prevent further lipoperoxidation. Samples were treated with 3 ml of 0.04 M H_2SO_4 and 2 ml of 0.8% thiobarbituric acid (TBA) in 0.1 M NaOH and then boiled for 40 min. at 100 °C. After rapid cooling, 4 ml of n-butanol were added and the mixture was centrifuged at 1000 xg for 10 min. TBARS formation was determined in the organic layer with a spectrophotometer (DU 604, Beckman, USA) at 535 nm. TBARS content was calculated from a standard curve of 1, 1', 3, 3'-tetraetoxypropane. All experiments were carried out in triplicate.

Subchronic oral toxicity

A total of 80 adult Wistar rats (40 male and 40 female) were randomly allotted to the control (12% Tween 80) and to 3 different DHC-treated groups. The animals were placed in plastic cages (5 animals/cage). The selected doses were 25, 50 and 100 mg/kg body weight. The animals were treated daily by gavage with the drug, dissolved in 12% Tween 80, for 35 days always at the same time and following the same procedure. The toxicological effects were assessed as follows. Body weight, water and food intake, and excretion output (urine/excrement) were recorded daily. At the end of the experiment the animals were fasted for 12 h and a urine sample was obtained from each animal. Urine samples were analysed for protein, glucose, ketone bodies, pH, specific gravity and red blood cells using a semi-quantitative kit (Ames® type I). At the same time, blood samples were withdrawn by puncture of the retroorbital plexus as described by Kraus (17). Haematological values such as red blood cells (RBC), haemoglobin (Hb), haematocrit, mean corpuscular volume, and white blood cells (WBC) were determined in heparinized blood samples using an automated cell meter (CC-510, CELM, Brazil). In addition, non-heparinized blood was allowed to coagulate and centrifuged. Serum biochemical parameters such as glucose, uric acid, albumin, globulin, total proteins, alkaline phosphatase, γ-glutamyl transpeptidase (γ-GT), aspartate aminotransferase (AsAT), alanine aminotransferase (ALAT), triglycerides, cholesterol and total lipids were determined immediately using an automated biochemical analyser (SBA-200, CELM, Brazil). After blood sampling, animals were killed and liver, kidneys, lungs, spleen, heart, adrenal glands, stomach and seminal vesicles or ovary/uterus were removed, blotted free of blood and weighed immediately on a digital scale.

Since significant differences were observed at the end of the experiment in the liver weights of the treated animals compared to the controls, four animals per group (control, and DHC 50 and 100 mg/kg) were selected randomly and a portion of the median lobe of the liver was withdrawn for histopathological analysis as described by Yoshitake (18). Liver cell alterations observed as induction of turbid tumefaction, microvacuolate degeneration and nuclear alterations were graded as follows: (-) 0- 20%, (+) 20- 40%, (++) 40- 60%, (+++) 60- 80%, (++++) 80- 100%.

Drugs

The following drugs were used: cimetidine (Tagamet® SmithKline), lanzoprazole (Ilzatec® Boehringer Ingelheim), Tween 80®(Synth, Brazil) and HEPES. All the other chemicals were purchased from Sigma Chemical Co, U.S.A. or similar. The kits used for haematological and biochemical analyses were from CELM, Brazil.

Statistical analysis

The statistical significance was assessed by one way analysis of variance (ANOVA). When the F value was significant, post hoc differences were determined by the Dunnett or Fisher test. Differences were considered significant if $P < 0.05$. All statistical analyses were performed using the Statistica 5.1 software (StatSoft, Inc.).

Results

Oral administration of the HCl/ethanol solution to the control group clearly produced the expected characteristic zonal necrotizing mucosal lesions. As shown in Figure 1, the ulcerative index was significantly lower in the DHC-treated group (100 and 200 mg/kg) compared to the control. DHC at the dose of 100 mg/kg p.o. exerted about 48% of protective effect in this pharmacological model. Interestingly, no significant differences were observed between the groups treated with DHC 100 mg/kg and 200 mg/kg or between the DHC 50 mg/kg group and the controls. In addition, lanzoprazole presented a protective effect of about 65% with a dose of 20 mg/kg or five times less.

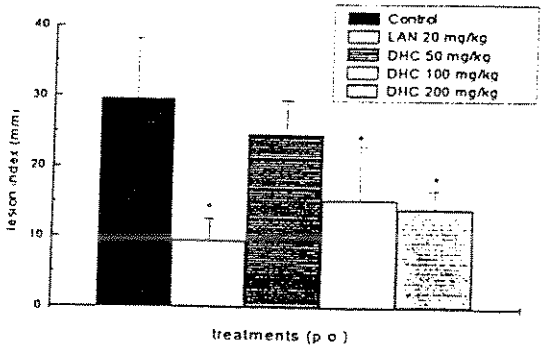

Figure 1. Effects of lansoprazole (LAN) and DHC on HCl/ethanol-induced gastric ulcers in mice. Results are expressed as mean ± SD (n=7-8). Asterisks indicate a significant difference from controls (ANOVA followed by Fisher's test). * $P < 0.001$.

The oral administration of DHC (100 mg/kg) effectively inhibited gastric lesions induced by cold-restraint stress, showing an efficacy similar to that of cimetidine at the same dose. The anti-ulcerogenic effect of DHC and cimetidine is presented in Figure 2. These results confirmed those previously obtained with rats (8).

Table 1. Body weight gain (g) of rats after oral treatment with DHC for 35 days.

Treatment	Dose (mg/kg)	*Males*			*Females*		
		N	initial	final	N	initial	final
Control		10	127.7 ± 10.4	280.0 ± 14.7	9	105.3 ± 8.1	194.3 ± 9.6
DHC	25	10	123.9 ± 8.3	282.3 ± 13.7	8	104.4 ± 8.3	185.6 ± 10.3
	50	10	120.1 ± 14.7	266.4 ± 10.2	9	102.0 ± 10.3	178.6 ± 15.5
	100	10	121.6 ± 7.2	266.0 ± 17.7	10	104.8 ± 7.3	185.8 ± 12.4

Values are expressed as mean ± SD. ANOVA Male $F_{(3,36)}$ for initial values= 0.99 $P > 0.05$; for final values = 1.71 $P > 0.05$
ANOVA Female $F_{(3,32)}$ for initial values = 0.27 $P > 0.05$; for final values = 2.50 $P > 0.05$

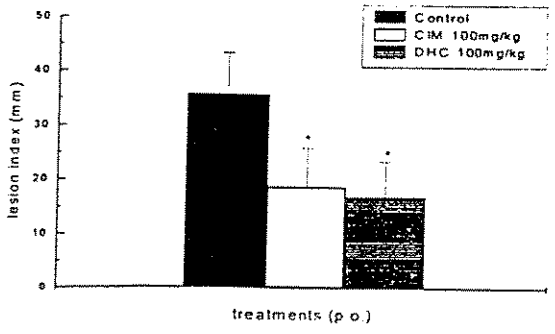

Figure 2. Effects of cimetidine (CIM) and DHC on hypothermic restraint stress-induced gastric ulcers in mice. Results are expressed as mean ± SD (n=8). Asterisks indicate a significant difference from controls (ANOVA followed by Dunnet's test). * $P < 0.001$.

Any possible effect of DHC (100, 200 and 300 μM) in removing free radicals in rat liver mitochondrias was also studied. The TBARS formation determined in the organic layer by a spectrophotometric method was not modified by DHC addition to the reaction medium. This result indicated that DHC was unable to remove the free radicals formed in the medium (data not shown).

No animal mortality due to toxicity and no significant differences in body weight gain were observed at the end of the 35 day subchronic oral toxicity experiment (Table 1). Food and water intake and urine/excrement output did not differ between treated animals and control during the period of treatment (data not shown). Furthermore, the average weights of most vital organs and visceral conditions were normal and comparable to the controls, except for a significant and dose-dependent increase in liver weight detected in both male and female rats (Figure 3). At the end of the 35 day treatment with oral DHC, no differences in urinary values were observed (results not shown).

Concerning the haematological parameters (Table 2), we found a reduction of RBC in male animals at the dose of 100 mg/kg. Although the 35 day DHC treatment did not elicit changes in most of the evaluated serum biochemical parameters, a significant reduction in serum alkaline

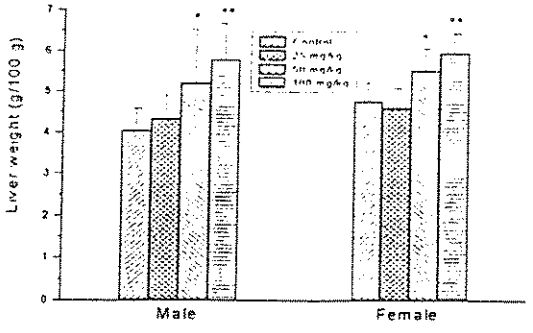

Figure 3. Effects of subchronic (35 days) oral treatment with DHC on rat liver weight. Results are expressed as mean ± SD (n=80). Asterisks indicate a significant difference from controls (ANOVA followed by Fisher's test). * $P < 0.05$; ** $P < 0.01$.

phosphatase and cholesterol, as well as an increase in γ-GT levels were observed in female rats. These results are presented in Table 3.

Finally, histopathological examination of the livers showed a marked dose- and sex-dependent damage characterized by turbid tumefaction, microvacuolate degeneration and nuclear

Table 2. Haematological parameters of rats after oral treatment with DHC for 35 days.

Treatment	Dose (mg/kg)	*Males* N	WBC ($x 10^3$)	RBC ($x 10^6$)	Hb (g %)	*Females* N	WBC ($x 10^3$)	RBC ($x 10^6$)	Hb (g %)
Control		10	11.5 ± 2.5	8.4 ± 0.4	16.8 ±0.9	9	11.0 ± 2.9	7.5 ± 0.8	14.9 ± 1.5
DHC	25	10	11.2 ± 2.0	8.3 ± 0.7	16.1 ± 0.9	8	11.4 ± 3.0	7.9 ± 0.7	15.5 ± 0.9
	50	10	12.0 ± 2.7	8.1 ± 0.7	15.9 ± 1.0	9	11.8 ± 3.4	7.9 ± 0.4	15.7 ± 0.7
	100	10	11.4 ± 2.3	7.7 ± 0.7*	15.3 ± 1.5*	10	10.3 ± 1.7	7.5 ± 0.7	14.8 ± 1.6

Values are expressed as mean ± SD. ANOVA Male $F_{(3,36)}$ for WBC = 0.23 P > 0.05; for RBC = 3.10 P < 0.05; for Hb = 3.18 P < 0.05 ANOVA Female $F_{(3,32)}$ for WBC = 0.51 P > 0.05; for RBC = 1.26 P > 0.05; for Hb = 1.31 P > 0.05 Fisher's test (in relation to control): * P < 0.005.

morphological alterations such as empty nuclei, nuclear membrane thickening, hyperchromatism, pycnosis, karyolysis and kariorrhexis (Table 4).

Discussion

Since there have been no reports of the anti ulcer activity of DHC, these possible effects are currently being examined using various ulcer models to obtain the anti-ulcer profile of these compounds. Recently, we reported a potent inhibitory activity of DHC against ethanol-, stress- and pylorus ligature-induced ulcerogenesis in rats. We speculated that the antiulcer effect DHC was not related to an increase in mucus sinthesis and/or mucus retention, but was probably due to an increase in mucosal defensive mechanisms such as prostaglandin production (8).

In the present study, two new gastric ulcer models were used to examine the ulcer-preventive effect of DHC in mice. Gastric lesion induced by cold restraint was used because it closely resembles the gastritis induced by psychological stress observed in man (19). Another common cause of gastric damage is excessive alcohol consumption (20). Thus, the cytoprotective property of DHC was also investigated in this last ulcer model.

There is an important discussion about the antiulcer compounds and their "truly" antiulcerogenic properties. One drug can be considered as a true antiulcer agent if the pre-exposure to "weak" gastric irritants such as low concentrations of ethanol (21), NaCl (22) or HCl (23) would also prevent the hemorrhagic injury induced, for example, by high concentration of intragastric ethanol (24). The fact that mild irritants prevent gastric necrosi is called adaptive cytoprotection (25) and seems to be mediated by prostaglandins (26, 27). But this is not the case for DHC. DHC can not to be considered as a "weak" irritant because this drug has shown antiulcerogenic actions on gastric lesions induced by severe irritants such as hypothermic-restraint stress, 100% ethanol and pylorus ligature.

Table 3. Serum levels of alkaline phosphatase, γ-glutamyl transpeptidase and cholesterol in female rats after oral treatment with DHC for 35 days.

Treatment	Dose (mg/kg)	N	Alkaline Phosphatase (IU/l)	γ-GT (IU/l)	N	Cholesterol (mg/dL)
Control		9	102.2 ± 20.9	0.22 ± 0.4	8	164.0 ± 37.8
DHC	25	8	75.3 ± 28.1	0.12 ± 0.3	-	-
	50	9	70.7 ± 13.7 *	0.33 ± 0.5	-	-
	100	10	69.9 ± 12.1 **	1.6 ± 1.7 *	8	103.9 ± 23.7 *

Values are expressed as mean ± SD. ANOVA $F_{(3,32)}$ for alkaline phosphatase = 5.76 P < 0.05; for γ-glutamyl transpeptidase = 4.75 P < 0.05; $F_{(1,14)}$ for cholesterol = 5.61 P < 0.05; Fisher's test (in relation to control): * P < 0.05; * * P < 0.01.

The ulcerative index was significantly lower in the DHC-treated group (100 and 200 mg/kg) compared to the control in the HCl/ethanol induced gastric lesions in mice. DHC at the dose of 100 mg/kg p.o. presented a protection of about 50% in this pharmacological model. Interestingly, no significant differences were observed between the groups treated with DHC 100 mg/kg and 200 mg/kg or between the DHC 50 mg/kg group and the controls. In addition, lanzoprazole presented about a 65% protective effect with a dose of 20 mg/kg or five times less. The cytoprotective action of some antiulcer drugs and the action of mild irritants are mediated by the action of endogenous prostaglandins known to play important role in maintaining mucosal integrity and to protect the gastric mucosa against various damaging agents (9, 23, 24, 27). The potent action of lanzoprazole on this model was not only due to a strong inhibition of gastric acid secretion. It was also reported that proton pump inhibitors have cytoprotective properties in addition to their antisecretory activity (28). Beside, effect of lanzoprazole is better than that of omeprazole because the latter drug has a longer plasma elimination half-life (29).

The oral administration of DHC effectively inhibited ulcer formation in the hypothermic-restraint stress model, showing a similar efficacy to cimetidine at the same dose. The results obtained with this model in mice confirmed those previously obtained with rats (8). Disturbances of gastric mucosal microcirculation, alteration of gastric secretion and abnormal motility have been considered to be the pathogenic mechanisms responsible for stress-induced gastric mucosal lesions and gastric mucus depletion (30). However, the most important factor in the genesis of stress ulcer is the increase in gastric acid secretion, which is often termed as the "aggressive factor" (31). The effects of DHC and cimetidine on this model are certainly due to their antisecretory properties.

It is well known that antioxidants can prevent some gastric lesions such as ethanol-induced gastric injury (9). We previously determined that DHC inhibits ethanol-induced gastric injury in rats (8) and ethanol/HCl gastric lesions in mice. Thus, we now investigated any possible effect of DHC in removing free radicals, i.e., its oxygen radical scavenger property in rat liver mitochondria.

Several antiulcerogenic compounds occurring in plants such as flavonoids are antioxidants and therefore may also act as scavengers of superoxide and other active oxygen species which have been shown to be destructive agents in inflammatory processes (32). The TBARS formation determined in the organic layer by a spectrophotometric method was not modified by DHC addition (100, 200 and 300 µM) to the reaction medium. The absence of significant effects in the rat liver mitochondrial lipid peroxidation assay suggests that the antiulcerogenic mechanism of DHC is not due to protection against free radical formation in the gastric mucosa.

Thus, all results obtained with DHC suggest both inhibition of gastric acid secretion and an increased prostaglandin secretion.

We knew that DHC has a low acute toxicity in mice (DL_{50} = 876 mg/kg) when administered by the oral route (8). In view of this finding, we also studied the subchronic (35 days) oral toxicity of DHC in rats. Subchronic oral treatment with DHC did not induce any obvious alteration in body weight gain or in other gross metabolic parameters such as water and food consumption or excretion in treated animals. Nevertheless, a marked dose-dependent liver hypertrophy accompanied by severe histopathological changes that are recognized markers of liver toxicity was found. The biochemical differences between the control and treated groups confirmed the suggested hepatotoxicity. Although the statistical significance was not reached in the male animals, an obvious tendency similar to that found in the female rats, was also observed. It is well known that some liver disorders such as cirrhosis and toxic hepatitis could be accompanied by decreases in erythrocyte count, haemoglobin and hematocrit (33).

Our findings of a reduction in RBC and Hb in male rats after subchronic treatment (Table 2) are in agreement with the hepatic alterations induced by DHC administration. We have no explanation for the marked differences in hepatotoxicity between males and females, but we suggest that they are related to the toxic mechanism of DHC. In addition, some studies have shown a sex-dependent expression of some cytochrome P-450 isoforms involved in drug metabolism that could modify the toxicity in a sex-dependent manner such as furano diterpenoids (34, 35). Several compounds occurring in plants have been shown to possess anti-ulcer properties (36). Diterpenelactones from *C. sublyratus*, another *Croton* species, were proved to be antiulcerogenic

compounds although with a reduced yield and a lower anti-ulcer potency compared to DHC (1, 37).

Table 4. Hepatic histopathological changes induced in rats by oral treatment with DHC for 35 days.

Animals	Dose (mg/kg)	Turbid Tumefaction					Microvacuolate Degeneration					Nuclear Iterations				
		-	+	++	+++	++++	-	+	++	+++	++++	-	+	++	+++	++++
	--	4					4					4				
Males	50			4				1	3				4			
	100				4					4				4		
	--	4					4					4				
Females	50				4				1	3				4		
	100					4					4				4	

In conclusion, DHC presents both protective and antisecretory activities. Our previous results (8) and those presented here clearly supported this statement. Although DHC is a compound with anti-ulcerogenic properties, its long-term use could induce hepatotoxicity. The mechanism of the antiulcer activity of DHC was not demonstrated by the present data. Further studies will be carried out to establish the mechanism of the antiulcer activity of DHC as well as to determine the cellular endpoint of its toxicity. Changes in the chemical structure of DHC have been made while maintaining its pharmacological properties and abolishing is toxic liver effects (unpublished data).

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C A HIRUMA-LIMA J S GRACIOSO D S NUNES A R M SOUZA BRITO
Effects of an essential oil from the bark of Croton cajucara Benth. on experimental gastric ulcer models in rats and mice

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Effects of an Essential Oil from the Bark of Croton cajucara Benth. on Experimental Gastric Ulcer Models in Rats and Mice

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Abstract

Croton cajucara Benth. (Euphorbiaceae) is widely used in Amazonian folk medicine for the treatment of a wide range of gastrointestinal symptoms. The essential oil from its bark was investigated for acute toxicity in mice and for its ability to prevent the formation of ulceration of the gastric mucosa in different models of experimentally induced gastric ulcer in mice and rats.

When previously administered orally at a dose of 100 mg kg^{-1}, the essential oil significantly reduced ($P < 0.01$) the gastric injury induced by hypothermic restraint stress (48%), indomethacin (47%), ethanol (86%) and pylorus ligature models (87%) in rats. In the HCl/ethanol-induced gastric ulcer model in mice, at oral doses of 100 and 200 mg kg^{-1}, the essential oil from *C. cajucara* significantly reduced ($P < 0.01$) the formation of gastric lesions by 52% and 76%, respectively, when compared with the control group. In rats submitted to pylorus ligature, the essential oil given orally increased the volume of gastric juice when compared with the control group ($P < 0.01$). In previous studies, when the essential oil (100 mg kg^{-1}) was administered intraduodenally to mice, no significant modifications were found in gastric parameters such as pH and total acid content after oil treatment. However, systemic alterations were observed. Here we observed significant changes ($P < 0.01$) in gastric juice parameters such as an increase in volume and a decrease in gastric acidity (pH and total acid content). The acute toxicologic effects of the essential oil from *C. cajucara* were assessed in mice. The LD50 values were 9.3 g kg^{-1} by the oral route and 680 mg kg^{-1} by the intraperitoneal route.

The good yield of essential oil obtained from dried *C. cajucara* bark (1%) as well as its anti-ulcerogenic activity and low toxicity suggest that pharmacological studies of this substance as a potential new anti-ulcerogenic drug are warranted.

An aromatic bitter tea made from the bark and leaves of *Croton cajucara* Benth. (Euphorbiaceae), commonly known as sacaca, is widely used in Amazonian folk medicine for the treatment of a wide range of gastrointestinal symptoms (Di Stasi et al 1989). We recently reported the anti-ulcerogenic activity of *trans*-dehydrocrotonin (DHC), the principal furane diterpene isolated from *C. cajucara* bark, in different ulcerogenic models in mice and rats (Rodriguez et al 1998; Souza Brito et al 1998) and later described the possible anti-ulcerogenic mechanisms involved in the action of DHC (Hiruma-Lima et al 1998). The acute and subchronic toxicity of this compound was also studied by our group in in-vivo and in-vitro assays that showed it to possess relatively low oral toxicity when administered for a short period of time (35 days).

In addition to DHC, the bark also contains 1% of a very pleasant essence composed principally of sesquiterpenes. Thus, in the present pharmacological studies we analysed the anti-ulcerogenic activity of this essential oil in indomethacin-, hypothermic restraint stress-, ethanol- and pylorus

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ligature-induced gastric ulcer in mice and rats. In the HCl/ethanol model in mice, we studied whether the effect of the essential oil was dose-dependent and compared it with that of lanzoprazole and omeprazole. Parameters of gastric acid secretion such as gastric acidity and gastric juice volume were analysed in animals submitted to the Shay method, which were treated with essential oil by the oral and intraduodenal route. Moreover, the in-vivo acute toxicological effect of the essential oil from the bark of *C. cajucara* was also determined.

Material and Methods

Animals

Male Wistar rats (250–300 g) and male albino Swiss mice (25–35 g) from the Central Animal House of the Universidade Estadual de Campinas (CEMIB/UNICAMP) were used. The animals were fed a certified Nuvilab CR-a (Nuvital) diet and had free access to water under standard conditions of illumination (12 h dark–12 h light), humidity (60 ± 1·0%) and temperature (21·5 ± 1·0%). Fasting was used before all assays because standard drugs or essential oil were administered orally (by gavage) or intraperitoneally using as vehicle a 12% solution of Tween 80 (10 mL kg^{-1}). All the protocols were approved by the Ethics Committee of UNICAMP (registered in the Brazilian National Council of Health – Res. 196/96). All the experiments were conducted in agreement with the recommendations of the Canadian Council on Animal Care (Olfert et al 1993).

Drugs

The following drugs were used: cimetidine, lanzoprazole, Tween 80 and indomethacin. All reagents were of a high grade of purity. The substances and reagents were prepared immediately before use.

Preparation and analysis of the essential oil

The stem bark of *Croton cajucara* Benth. was collected at our experimental plantation in Benfica, near Belém, Pará, Brazil. A voucher specimen (number 247) has been identified by Nelson A. Rosa and deposited in the IAN Herbarium in Belém, Brazil. The air-dried and milled bark (20 kg) was subjected to steam distillation for 6 h, a first fraction of 163 mL (F1) was collected after 3 h and a second fraction of 42 mL (F2) at the end of the process. Preliminary GC-FID and GC-MS analyses performed with an Hewlet Packard system using a HP-5 capillary column showed very similar patterns for both F1 and F2 fractions, which were composed mainly of $C_{15}H_{24}$ sesquiterpenes. α-Copaene (20·9%) and cyperene (29·0%) were the main components of F1, as confirmed by ^{13}C NMR spectra measured in a Varian spectrometer operating at 75·4 MHz and using benzene-d_6 as solvent. Complete analyses of the samples are in progress. The F1 fraction was used for the pharmacological tests. F1 was emulsified in 12% Tween 80 before administration to the animals.

In-vivo toxicity

The acute oral toxicity of essential oil from *C. cajucara* bark was determined in male albino Swiss mice which had been fasted for 12 h. Increasing doses of essential oil were administered orally by gavage to groups of 10 animals for each dose level (1, 2·5, 5 and 7·5 g kg^{-1}). Animals receiving the vehicle (12% Tween 80) served as control. The groups were observed at 0, 30, 60, 120, 180 and 240 min after oil administration and then twice a day for the next 14 days. At the end of this period the number of survivors was recorded and the acute toxicologic effect was determined on the basis of mortality, expressed as LD50. The oral LD50 value was obtained using a software based on the method of Litchfield & Wilcoxon (1949).

Anti-ulcerogenic activity

HCl/ethanol-induced ulcer. The anti-ulcerogenic activity of essential oil in HCl/ethanol-induced gastric ulcer was assessed in mice as described by Mizui & Doteuchi (1983). Mice were divided into groups of 6–12 animals and fasted for 24 h before receiving an oral dose of either the vehicle, 12% Tween 80 (10 mL kg^{-1}), lansoprazole (20 mg kg^{-1}), omeprazole at the same dose or essential oil (50, 100 or 200 mg kg^{-1}). After 50 min all groups were orally treated with 0·2 mL of a 0·3 M HCl/60% ethanol solution (HCl/ethanol) for gastric ulcer induction. Animals were killed with ether 1 h after the administration of HCl/ethanol, and the stomachs were excised and inflated by injection of saline (2 mL). The stomachs were fixed in 5% formalin for 30 min and opened along the greater curvature. The extension of the lesions was measured, and the ulcerative index is expressed as the sum of all lesions.

Indomethacin ulcer. A total of 17 rats were randomly divided into 3 groups and fasted for 24 h, with free access to water before the experiment.

Thirty minutes after oral administration of essential oil (100 mg kg^{-1}), cimetidine (100 mg kg^{-1}) or 12 % Tween 80 (10 mL kg^{-1}), 30 mg kg^{-1} of indomethacin was subcutaneously administered to unanaesthetized rats from each group according to the method of Hayden et al (1978). Indomethacin was dissolved in 5% sodium bicarbonate. The animals were killed 4 h later, the stomachs removed and opened and the gastric lesions were determined as described above.

Hypothermic restraint stress ulcer. The anti-ulcerogenic activity of *C. cajucara* essential oil was assessed in the hypothermic restraint stress-induced gastric ulcer model in rats according to the method of Levine (1971), with some modifications. Rats were divided into groups of 7 animals each. After 24 h of starvation, the animals received an oral dose of essential oil (100 mg kg^{-1}), cimetidine (100 mg kg^{-1}) or 12% Tween 80 (10 mL kg^{-1}). One hour after the treatment, gastric ulceration was induced by immobilising the animals inside a closed cylindrical cage maintained at 4°C. After 3 h the animals were killed with ether and the stomachs removed and examined for ulcers as described previously.

Ethanol-induced ulcer. The ethanol-induced ulcer assay was carried out in rats according to the method of Morimoto et al (1991). A total of 17 animals were randomly divided into 3 groups and fasted for 24 h, with free access to water before the experiment. One millilitre of 99·5% ethanol was orally administered to the animals which 1 h previously had been treated with essential oil (100 mg kg^{-1}), omeprazole (20 mg kg^{-1}) or 12% Tween 80 (10 mL kg^{-1}). One hour after ethanol administration the animals were killed, the stomachs were removed and opened and the ulcerative index was determined.

Shay ulcer. A total of 18 rats were randomly divided into three groups and fasted for 24 h, with free access to water. Thirty min after oral administration of essential oil (100 mg kg^{-1}), cimetidine (100 mg kg^{-1}) as positive control or vehicle (12% solution of Tween 80, 10 mL kg^{-1}), a pylorus ligature was performed as described by Shay et al (1945). The animals were killed 4 h later, the abdomen was opened and another ligature was placed around the oesophagus close to the diaphragm. The stomach was removed, inspected internally, and its contents drained into a graduated centrifuge tube and centrifuged at 2000 rev min^{-1} for 10 min. The supernatant volume and pH were recorded with a digital pH meter. The total acid content of gastric secretion was also determined by titration to pH 7·0 with 0·05 N NaOH using a digital burette. Gastric lesions were evaluated by examining the inner gastric surface with a dissecting binocular microscope and the mucosal lesions were counted and scored as described above.

Determination of gastric secretion. A total of 18 mice were randomly divided into three groups and fasted for 24 h with free access to water. The assay was performed by the method of Shay with some modifications (Shay et al 1945). Immediately after pylorus ligature, *C. cajucara* essential oil (100 mg kg^{-1}), cimetidine (100 mg kg^{-1}) as positive control or vehicle, a 12 % solution of Tween 80 (10 mL kg^{-1}), was administered intraduodenally. The animals were killed 3 h later and the same procedures as described for Shay ulcer were followed.

Statistical analysis

Results are expressed as the mean ± s.d. Statistical significance was determined by one-way analysis of variance followed by Dunnett's test, with the level of significance set at $P < 0.05$.

Table 1. Effects of omeprazole, lanzoprazole and different doses of essential oil from *Croton cajucara* on HCl/ethanol-induced gastric ulcer in mice.

Treatments (p.o.)	Dose (mg kg^{-1})	N	Ulcerative Index (mm)	Inhibition (%)
Control	–	12	32·5 ± 8·37	–
Omeprazole	20	8	17·5 ± 7·62*	46
Lansoprazole	20	7	9·57 ± 3·10*	71
C. cajucara oil	200	7	10·6 ± 2·82*	67
C. cajucara oil	100	6	15·7 ± 2·88*	52
C. cajucara oil	50	7	30·1 ± 10·2	7

Results are expressed as mean ± s.d. Analysis of variance: $F(5·41) = 17·1$ $P < 0·05$. Dunnett's test *$P < 0·01$.

Results

The LD50 value obtained by probit analysis for oral administration of *C. cajucara* essential oil was 9·26 g kg^{-1} ($r = 0·99 \pm 0·24$, $n = 10$, $P > 0·05$). These data are not shown.

The effects of essential *C. cajucara* oil on induced gastric ulcer were first investigated in mice and the results are shown in Table 1. Oral administration of HCl/ethanol solution to the control group clearly produced the expected characteristic zonal necrotizing mucosal lesions. Essential oil was given orally at doses of 50, 100 and 200 mg kg^{-1}

while omeprazole and lanzoprazole (positive controls) were administered orally at a dose of 20 mg kg^{-1}. The lesion index for the control group of the HCl/ethanol-induced gastric ulcers was 32·5 ± 8·37 mm. The anti-ulcer drugs lanzoprazole and omeprazole and essential oil (200 and 100 mg kg^{-1}) significantly inhibited ulcer formation by 71, 46, 67 and 52%, respectively. Interestingly, no significant differences were observed between the groups treated with 100 or 200 mg kg^{-1} of essential oil or between the group treated with 50 mg kg^{-1} of essential oil and the negative control.

The effects of essential oil on the four assayed methods of induced gastric lesions are shown in Table 2. Oral administration of *C. cajucara* essential oil at a dose of 100 mg kg^{-1} inhibited the appearance of gastric lesions induced by hypothermic restraint stress, indomethacin, ethanol and pylorus ligature. The best inhibitory effect on the ulcerative index was observed in the model of pylorus ligature (87%) followed by the ethanol model (86%). The same relative potency (47% inhibition) was observed for indomethacin- and hypothermic restraint stress-induced gastric lesions.

In the pylorus ligature method, the administration of the essential oil by different routes produced a significant modification in gastric volume, pH and gastric acid content (Table 3). The pylorus-ligated rats treated with essential oil (100 mg kg^{-1}, p.o.) only showed a significant increase in gastric volume compared with the control group. In contrast, cimetidine at 100 mg kg^{-1} significantly reduced gastric-juice volume and acidity and increased gastric pH. However, the essential oil administered intraduodenally to mice at same dose was effective in inducing a significant increase in gastric juice and pH, and a decrease in total gastric acid.

Discussion

Since no studies of the anti-ulcer activity of *Croton cajucara* are available, the possible effects of this

Table 2. Effects of essential oil from *Croton cajucara* on the four assayed methods of induced gastric lesions in mice and rats.

Animal	Gastric lesion model	Treatment	N	Ulcerative index (mm)	Inibition (%)
Rats	Indomethacin	Control	6	66·0 ± 14·8	–
		Cimetidine	6	6·0 ± 2·6*	91
		Essential oil	5	34·9 ± 9·3*	47
	Ethanol	Control	5	91·8 ± 12·4	–
		Omeprazole	6	32·2 ± 14·3*	65
		Essential oil	6	12·8 ± 4·4*	86
Mice	Ligature	Control	6	17·3 ± 5·43	–
		Cimetidine	5	2·33 ± 2·07	86
		Essential oil	5	2·20 ± 2·28*	87
	Stress	Control	7	40·6 ± 11·6	–
		Cimetidine	7	21·1 ± 2·54*	48
		Essential oil	7	21·4 ± 4·61*	47

Cimetidine or essential oil from *C. cajucara* were administered orally at a dose of 100 mg kg^{-1} and omeprazole was given orally at a dose of 20 mg kg^{-1}. Values for ulcerative index are expressed as mean ± s.d. Analysis of variance gave indomethacin: $F_{(2\cdot14)} = 51\cdot2$ $P < 0\cdot05$; ethanol: $F_{(2\cdot14)} = 72\cdot6$ $P < 0\cdot05$; ligature: $F_{(2\cdot14)} = 32\cdot5$ $P < 0\cdot05$; stress: $F_{(2\cdot18)} = 15\cdot9$ $P < 0\cdot05$. Dunnett's test $^*P < 0\cdot01$.

Table 3. Effects of essential oil from *Croton cajucara* on biochemical parameters of the gastric juice obtained from pylorus-ligated rats and mice (n = 6).

Animals	Treatments	Route	pH	Gastric juice volume (mL)	Total gastric acid (mEq mL^{-1})
Rats	Control	p.o.	3·21 ± 1·15	2·50 ± 0·77	1·16 ± 0·61
	Cimetidine	p.o.	6·33 ± 1·03**	1·43 ± 0·39**	0·31 ± 0·12**
	Essential oil	p.o.	2·80 ± 0·84	3·90 ± 0·44**	0·64 ± 0·20
Mice	Control	i.d.	3·66 ± 0·52	0·36 ± 0·03	6·02 ± 1·58
	Cimetidine	i.d.	6·13 ± 0·73**	0·65 ± 0·23*	1·87 ± 0·62**
	Essential oil	i.d.	7·17 ± 0·82**	0·72 ± 0·28**	1·36 ± 0·64**

Cimetidine were administered orally (p.o.) to rats or intraduodenally (i.d.) to mice at a dose of 100 mg kg − 1. Data are expressed as mean ± s.d. Analysis of variance for rats: $F_{(2\cdot15)}$ for pH = 20·5 $P < 0\cdot05$; $F_{(2\cdot15)}$ for volume = 24·4 $P < 0\cdot05$; $F_{(2\cdot15)}$ for total acid = 7·29 $P < 0\cdot05$. Analysis of variance for mice: $F_{(2\cdot15)}$ for pH = 39·9 $P < 0\cdot05$; $F_{(2\cdot15)}$ for volume = 5·0 $P < 0\cdot05$; $F_{(2\cdot15)}$ for total acid = 35·6 $P < 0\cdot05$. Dunnett's test $^*P < 0\cdot05$ and $^{**}P < 0\cdot01$.

plant are currently being investigated using various ulcer models to determine the potential anti-ulcerogenic profile of this Brazilian medicinal plant (Souza Brito & Nunes 1997).

Our group has previously demonstrated the anti-ulcerogenic properties of DHC isolated from the bark of *C. cajucara*. In the present study, we analysed another bark component possibly involved in the anti-ulcerogenic activity of this plant, namely the essential oil. Previous chromatograph analyses showed that the essential oil has no traces of DHC in its composition. Thus, the possible anti-ulcerogenic effect of essential oil was attributed to its own composition. The essential oil of *C. cajucara* bark did not present significant acute toxicological effects. The oral LD50 was obtained with a 90-fold higher dose than the active anti-ulcer dose ($100\,mg\,kg^{-1}$).

The present study was carried out using different experimental models of gastric ulcer which operate by distinct mechanisms of ulcerogenesis (Desai & Parmar 1994). The preventive effects of the essential oil of *C. cajucara* bark on gastric ulcer induced by the various necrotizing agents studied were investigated in mice and rats. The ulcerative index was significantly lower in essential oil-treated mice (100 and $200\,mg\,kg^{-1}$) compared with the control for HCl/ethanol-induced lesions. Essential oil at an oral dose of $100\,mg\,kg^{-1}$ presented a 50% protection in this pharmacological model. Interestingly, no significant differences were observed between the groups treated with oil at $100\,mg\,kg^{-1}$ and $200\,mg\,kg^{-1}$ or between the groups treated with essential oil at $50\,mg\,kg^{-1}$ and the control.

Hypothermic restraint stress ulcers have been widely used experimentally for the evaluation of anti-ulcer activity in rats because of data reproducibility (Murakami et al 1985). Disturbances of gastric mucosal microcirculation, alteration of gastric secretion and abnormal motility have been considered to be the pathogenic mechanisms responsible for stress-induced gastric mucosal lesions and gastric mucus depletion (Koo & Cho 1986). However, the most important factor in the genesis of stress ulcer is the increase in gastric acid secretion and this is often termed the aggressive factor (Goa & Monk 1987).

The essential oil ($100\,mg\,kg^{-1}$) significantly protected the gastric mucosa against hypothermic restraint stress-induced ulcers in mice with an effect comparable to that of cimetidine at the same dose.

Ethanol treatment induces solubilization of mucus constituents in the stomach with a concomitant fall in the transmucosal potential difference, and increases Na^+ and K^+ flux into the lumen, pepsin secretion, the loss of H^+ ions and the histamine content in the lumen. This drug also depresses tissue levels of DNA, RNA and proteins, leading to flow stasis in injured areas (Szabo 1987). Moreover, it is well known that ethanol-induced ulcers are not inhibited by anti-secretory agents such as cimetidine, but are inhibited by agents which enhance mucosal defensive factors such as prostaglandin E_2 (Robert et al 1979). In the present study, *C. cajucara* essential oil significantly protected the gastric mucosa against the injury induced by ethanol. These results further indicate that this essential oil may enhance gastric mucosal defensive factors such as mucus, prostaglandins, or both.

Anti-inflammatory agents such as indomethacin reduce gastric cyclooxygenase activity and decrease endogenous prostaglandin levels (Konturek et al 1984). These agents break the mucosal barrier, provoke an increase in gastric mucosal permeability to H^+ and Na^+ ions and a drop in the transmucosal potential difference, and induce the formation of erosions and ulcers (Droy-Lefaix 1988). There is mounting evidence that an increase of certain endogenous prostaglandins can enhance gastric mucosal resistance against ulcerogenic agents such as anti-inflammatory agents (Wallace & Whittle 1985). In this assay, the essential oil from *C. cajucara* was also able to produce a significant reduction of the gastric mucosal damage induced by indomethacin, indicating once again the probable local increase in prostaglandin synthesis. Subsequently we showed the biochemical results obtained after submitting the animals to pyloric ligature. Rats were pretreated orally with essential oil or cimetidine and mice were pretreated with the same doses by the intraduodenal route. The oral pretreatment with cimetidine provoked changes in the acidity and volume of gastric juice. The essential oil only provoked a marked increase in the volume of gastric juice. The intraduodenal administration of both drugs had similar effects on all of the other parameters analysed. Like cimetidine, the essential oil was also effective in reducing gastric acidity and in increasing the volume of gastric juice.

There is evidence of an involvement of prostanoids in the accumulation of fluid in the gastric lumen; prostaglandin E_2 causes a significant increase of volume flow in the stomach (Droy-Lefaix 1988). Moreover it seems that the essential oil of *C. cajucara* exerts a kind of cytoprotective action mainly through a systemic action because this action is present not only when the oil is administered orally, but also when it is administered intraduodenally. Thus, its protective effect does not depend on contact with the gastric mucosa.

The present results clearly indicate that oral administration of essential oil from *C. cajucara* bark produced a significant anti-ulcer effect that was probably due to an increase in mucosal defensive mechanisms such as prostaglandin production. Moreover, the low toxicity level observed in-vivo supports the acute medicinal use of this specie with a wide safety range and without severe risks for users.

In conclusion, the oral administration of *C. cajucara* essential oil displayed a significant anti-ulcer activity with no toxicological effects. New experiments are currently underway to determine the composition of the essential oil as well as the anti-ulcer mechanisms involved.

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ANTIULCEROGENIC MECHANISMS OF ESSENCIAL OIL FROM *Croton cajucara*

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Abstract

An aromatic bitter tea prepared from the bark of *Croton cajucara* Benth. (Euphorbiaceae) is widely used in Amazonian folk medicine for the treatment of a wide rang of gastrointestinal symptoms. We recently reported the antiulcerogenic activity of essential oil in different ulcers models in mice and rats such as hypothermic restraint stress, indomethacin, ethanol and pylorus ligature models. In the HCl/ethanol-induced gastric ulcer model in mice, the essential oil significantly reduced the formation of gastric lesions when compared to the control group. In rats submitted to pylorus ligature, the essential oil (p.o.) increased the volume of gastric juice when compared with the control group. No significant modifications where found in gastric parameters such as pH and total acid content after oil treatment. However, systemic alterations where observed when the essential oil was previously administered intraduodenally to mice such as an increase in volume and a decrease in gastric acidity. The acute toxicologic effects of the essential oil from *C. cajucara* were assessed in mice. The good yield of essential oil obtained from dried *C. cajucara* bark as well as its antiulcerogenic activity and low toxicity suggest the continued pharmacological studies of this substance as a potential new antiulcerogenic drug. Thus, in the present pharmacological studies we analysed the mechanisms involved in the antiulcerogenic activity of the essential oil. We studied the effects of essential oil on the ability of healing of acetic acid-induced gastric ulcer in rats were evaluated 14 days after acid injection. We also studied the ability of essential oil from *C. cajucara* to increase PGE_2 release from mucous cells of the rat stomach. The results shown that a single oral administration of essential oil (100 mg/kg) for 14 consecutive days accelerate the healing of chronic gastric ulcer (32%) in rats ($p<0.01$). In addition, the essential oil did not induce any alteration in gastric pH or body weight during the all treatment ($p>0.01$). We also observed that essential oil induced a significant increase in PGE_2 production by glandular cells (103 % in relation to control). The amount of gastric mucus raising two times higher than the control group ($p<0.01$). Thus, the protective and curative effect of essential oil from *C. cajucara* bark on induced gastric lesions was due to an increase of PGE_2 release that represents an increase of the protective factors of the gastric mucosal.

Introduction

Croton cajucara Benth., is a well-known medicinal plant used in Amazonian folk medicine to treat several illnesses such as diarrhoea, diabetes, liver inflammation an the control high cholesterol levels (Di Stasi et al, 1989; Simões et al, 1979; Carvalho et al 1996).

Among other medicinal uses, the stem bark of this plant is prepared as tea (only 5g bark in 100 ml water) to be drunk in cases of heartburn, gastritis and peptic ulcer (Souza Brito and Nunes, 1997). Thanks to its strong bitter taste the tea is not taken in large doses, but daily for long periods from two to eight weeks. The last studies reported the antiulcerogenic activity of *trans*-dehydrocrotonin (DHC) the principal furane diterpene isolated from *C. cajucara* bark (Souza Brito et al, 1998) and later described the possible antiulcerogenic mechanisms involved in the action of DHC (Hiruma-Lima et al, 1998a). But previous chromatograph analyses showed that the essential oil has no traces of DHC in its composition. Thus, the antiulcerogenic effect was last observed of essential oil was attributed to its own composition. Essential oil (up to 1.5% dry bark) containing primarily sesquiterpenes (Nunes et al, 1993). The bark oil was demonstrated in various pharmacological experiments and the oral administration of essential oil displayed a significant antiulcerogenic activity with no toxicological effects (Hiruma-Lima et al 1998b). Moreover, the aimed of this paper is to determine the antiulcer mechanisms involved with the essential oil from *C. cajucara*.

Methods

Animals. Male Wistar rats weighing 150-250 g from the Central Animal House of the Universidade Estadual de Campinas (CEMIB/UNICAMP) were used in all experiments. The animals were fasted prior to all assays because standard drugs or essential oil were always

administered orally (by gavage-10 ml/kg) using a 12% solution of Tween® 80 as vehicle. Animals received a certified Nuvilab CR-a® (Nuvital) diet and water *ad libitum* under standard conditions of 12 h dark-12 h light, humidity (55%) and temperature (22 ± 1 °C). The protocols were approved by the UNICAMP Institutional Animal Care and Use Committee which follows the recommendations of the Canadian Council on Animal Care (Olfert et al., 1993).

Drugs and Chemicals. The following drugs were used: cimetidine (Tagamet® SmithKline), Tween 80® (Synth, Brazil), indomethacin (Sigma Chem. Co, U.S.A.). The chemicals used in the nutritive solutions were all of analytical grade. The substances and reagents were prepared immediately before use.

Preparation of the essential oil. The stem bark of *Croton cajucara* Benth. was collected in experimental plantation in Benfica, near Belém, Pará, Brazil. A voucher specimen number 247 has been identified by Prof. Nelson A. Rosa and deposited in the IAN Herbarium in Belém, Brazil. The air dried and milled bark (20 kg) was subjected to steam distillation for 6 h. Preliminary GC-FID analysis performed in a HP-8969 system showed that the essential oil was composed principally by low oxygenated sesquiterpenes. Complete analyses of the samples are in progress. For the pharmacological tests here the essential oil was used and emulsified in 12% Tween 80®.

Acetic acid-induced gastric ulcers. The experiment were performed according to the method of Takagi et al. (1969). Male Wistar rats weighing 150 to 200 g at the time of operation were used in this experiment. The animals were deprived of food for 24hs because if the stomach is filled with the gastric content, the infection is slightly difficult and haemorrhage often occurred after removing the injection needle. All the experiments were performed between the hours of 09:00 and 11:00 h. Under ether anaesthesia laparotomy was performed through a midline epigastric incision. After exposing the stomach, 30% acetic acid, 0.05ml (v/v) per animal were injected into subserosal layer in the glandular part of anterior wall, with care taken not to disturb the blood vessels. At the injection of acetic acid solution a thumb was placed tightly on the inserted needle in order to avoid the leak of the solution. After injection, the needle was pull out, but the thumb was still placed on that position for at least 30 second to prevent the leak of acetic acid by removing of the needle. Then the stomach was bathed with saline to avoided adherent external surface of the ulcerated region that was strongly adherent to the liver which formed a part of the base of the ulcer so that this separation could not be performed without perforation of the stomach. Then the abdomen was closed and the animal was fed normally. Essential oil (100 mg kg^{-1}), cimetidine (100 mg kg^{-1}) or vehicle (10 ml kg^{-1}) was administered orally for 14 consecutive days, once a day, beginning 2 days after the surgery. Body weight was recorded daily at the same time during the all days of experiments. On the day following the last drug administration, the animals were sacrificed at proper intervals to assess the healing processes of the ulcer. The stomach was removed and the pH of the content of gastric secretion was recorded by PHmeter (Marconi, Brazil). The gastric lesions were evaluated by examining the inner gastric surface with a dissecting binocular microscope. Subsequently the ulcer area (mm^2) and curative ratio (%) were measured as described by Takagi et al. (1969).

Prostaglandin synthesis determination. The rats were deprived of food for 24 h prior to the experiment and all experiments were performed between 09:00 and 11:00 a.m. Groups consisting of at least 4 rats received one of the following solutions: 12 % Tween 80 solution (vehicle, p.o.), indomethacin (20 mg kg^{-1}, s.c.) used as a negative control, and essential oil (100 mg kg^{-1}, p.o.). Indomethacin was dissolved in 5% sodium bicarbonate solution, pH=8,3. Additional rats were treated with association of indomethacin, 30 min prior to treatment to essential oil. Thirty min after treatment, the animal were killed with ether and the abdomen was opened. The gastric mucus was excised and weighed. Then it is suspended in 1 ml of 10 mM sodium phosphate buffer, pH 7.4. The tissue was finely minced with a scissor and then incubated at 37°C for 20 min PGE_2 in the buffer was measured by enzymeimmunoassay (RPN222 - Armersham). The method was following as previously described by Curtis et al. (1995).

Statistical analysis. Results were expressed as mean ± SEM or SD. One-way analysis of variance followed by Fisher's test with the level of significance set at $p < 0.05$ was used in the other experiments. All statistical analyses were performed using the Statistica 5.1 software (StatSoft, Inc.).

Results

In general, animals withstood the method of the production of chronic gastric ulcer well and unfavourable symptom could not be observed. The body weigh of the treated animals with ulceration showed almost the same increase compared with the control group. However, 20% animals subjected to 30% acetic acid died by perforation on the 2nd and 5th day after the operation. This related for method was expected because the treatment and mainly the surgery (Takegi et all, 1969). Then the production and recovery process of these ulcers treated with essential oil (100 mg kg^{-1}) and cimetidine at the same dose, were examined in relation to the control group. The results are summarised in table 1. On the 14th day after the operation each animal from control group showed an apparent ulceration which had a punched-out appearance with a steep wall and the undermining of the floor. It was 20.3 ± 1.55 cm in size (figure 1-A). The floor of the ulcer was covered with necrotic material. In some stomach the external surface of the ulcerated region was adherent to the liver which formed a part of the base of the ulcer. The oral administration of cimetidine (100 mg kg^{-1}) and essential oil at the same dose for 14 consecutive days accelerated the healing of gastric ulcer in rats producing a 32.2 and 32.3 % cure rate respectively compared with the control group treated with vehicle (figure 1-B). This healing of gastric ulcer with that both drugs did not provoke an increase in pH values as also shown in table 1. No significant differences in body weight gain was observed at the end of the 14 day of assay (data not show). The data obtained for gastric PGE_2 production using EIA are shown in figure 2. In ours experiments, we had observed that the essential oil at dose of 100 mg kg^{-1} markedly increased the capacity of the gastric mucosa to synthesize mucus (figure 3).

Although, there was an increase of PGE_2 production by gastric tissue from essential oil, the gastric tissue from indomethacin (20 mg kg^{-1}, sc) treated rats showed a significant decrease. The increase in PGE_2 obtained in the treatment with essential oil was completely abolished by indomethacin. Thus, indomethacin clearly inhibits the PGE_2 increase induced by essential oil at dose of 100 mg kg^{-1}.

Table 1. Effect of Essential oil on the healing process of ulcers produced by the injection of 30% acetic acid solution into the rat stomach. The ulceration was observed on the 14th days after surgery.

Treatment (p.o)	N	Dose (mg/kg)	Lesion area (mm^2)	pH	Curative ratio %
Control	9	-	20.3 ± 1.55	2.06 ± 0.20	-
Cimetidine	8	100	13.7 ± 1.87 *	2.39 ± 0.37	32.2
essential oil	7	100	13.2 ± 0.38 *	2.50 ± 0.38	32.3

Expressed as mean ± SEM. Fisher's test, * $P < 0.05$.

Discussion

We have previously reported the antiulcerogenic activity of DHC, the principal furane diterpene isolated from *C. cajucara* bark, in different ulcerogenic models in mice and rats (Souza Brito et al., 1998; Rodriguez et al., 1998) and later described the possible antiulcerogenic mechanisms involved in the action of DHC (Hiruma-Lima et al, 1998a). In addition to DHC, the bark also contains 1% of a very pleasant essence composed principally of sesquiterpenes. Later, our groups assay the pharmacological studies to analysed the antiulcerogenic activity of this essential oil obtained from *C. cajucara* bark in indomethacin-, hypothermic restraint stress-, ethanol- and pylorus ligature-induced gastric ulcer in mice and rats. In the HCl/ethanol model in mice, we studied whether the effect of essential oil was dose-dependent and compared it with that of control-drug. The parameters of gastric acid secretion such as gastric acidity and gastric juice volume were analysed in animals submitted to the Shay method, which were treated with essential oil by the oral and intraduodenal route. Moreover, the *in vivo* acute toxicologic effect of the bark essential oil from *C. cajucara* was also determined (Hiruma-Lima et al 1998b).

Now we analysed the probably mechanisms involved with antiulcerogenic action of essential oil that appears seems different from DHC.

The ulcer produced by the injection of acetic acid solution into the rat stomach wall was assumed to be a so-called intractable ulcer which is frequently seen clinically but is seemed to be very difficult to produce in experimental

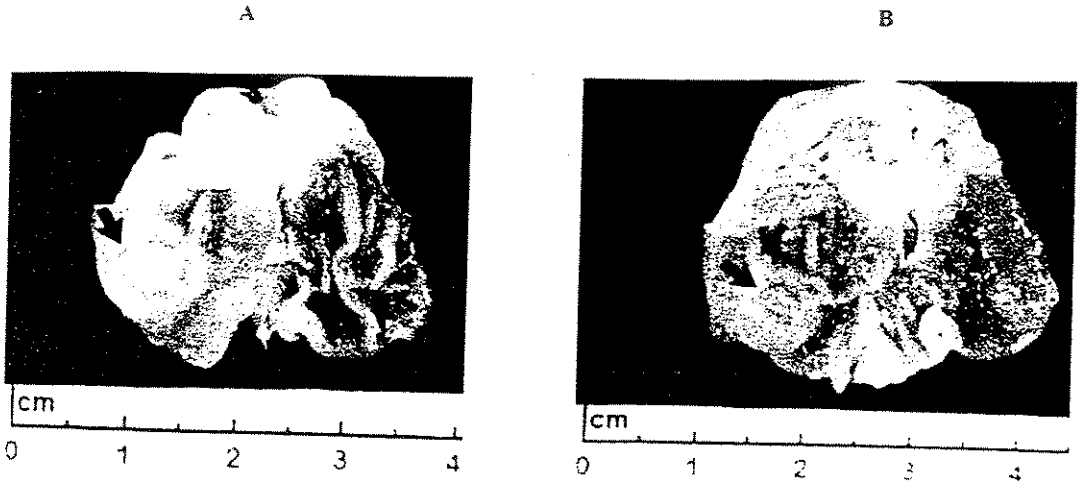

Figure 1 – Gastric ulcers produced by the injection of 30% acetic acid solution into the stomach walls in rats. Figure A and B shown representative stomachs of ulcerated rats with solution 12 % tween 80 and essential oil (100 mg/kg/day), respectively.

animals(Monfort et al, 1973). Two reasons were thought. The ulcer persisted more than 150 days without any additional procedure and the enlargement of the ulceration and the appearance of haemorrhages in the floor or on the edges of the lesions was confirmed during the healing process (Takagi at al, 1969). During the period up to 150 days after the infection of 30% acetic acid solution, the muscularis mucosae and muscle coat of the stomach were not repaired at all. The inhibition of gastric acid or pepsin did not accelerate the ulcer healing, and that the agents stimulating the epithelization or granulation of the stomach tissue exerted a significant acceleration of the repair in the early phase of the ulceration (Halter et al, 1995).

Ito et al (1994) indicated that cimetidine and omeprazole mainly accelerate the healing of chronic gastric ulcers in rats with a limited food intake time, by an increase in gastrin secretion, whereas the inhibition of acid secretion is little related to ulcer healing.

Skarstein (1996) reported an increased change of mucosal blood flow around the ulcer in his animal experiment, and suggested that more prostaglandins, which cause vasodilatation, were synthesised in the ulcer region rather than in other parts of the gastric mucosa. The large blood supply seems to reflect the active reepithelization, which requires an abundant supply of glucose and oxygen (Sato et al, 1995).

The results of the present study support the hypothesis that essential oil (100 mg kg^{-1}, p.o.) from C. *cajucara* Benth. increased the mucus synthesis and/or retention and increased the luminal PGE_2 concentration 2-fold over basal levels. Interesting results was obtained when the animals were treated with a combination of both drugs, essential oil and indomethacin. The increase in PGE_2 obtained in the treatment with essential oil was completely abolished by indomethacin, indicated that this last one clearly inhibits the PGE_2 raise induced by essential oil. This technique allows for the determination of the capacity of the tissue sample to generate PGE_2 from endogenous arachidonic acid and has been shown to correlate well with measurements of prostaglandin synthesis by gastrointestinal tissues (Curtis et al., 1995).

Several study shows a severe decrease in gastric mucosal blood flow after treatment with indomethacin, a typical NSAID, and a further decrease when HCl was topically administered on the mucosa (Kauffman, 1989). It seems likely that indomethacin inhibits cycloxygenases, resulting in the decreased mucosal prostaglandin levels and decreases mucosal blood circulation (Trevethick et al, 1995). Prostaglandin synthesis is critical to the maintenance of gastric mucosal integrity but the mechanism involved with the cytoprotective action of prostaglandins remains incompletely understood. Among the many factors that may contribute to the protective of mucus and bicarbonate secretion, maintenance of gastric blood flow during exposure to an irritant and inhibition of inflammatory mediator release from mast cells (Guth et al, 1984). Prostaglandins are also capable of modulation gastric

secretion. Gastric prostaglandin synthesis has been suggested to be modulated by a number of factors, including stress, luminal irritants and through activation of sensory afferent or cholinergic efferent nerves (Kato et al, 1997). An examination of the phytochemical literature for anti-ulcer molecules reveals that anti-ulcer activity is not confined to one class of compound and such activity is usually discovered by screening various plant molecules against one or more animals models (Lewis & Hanson, 1991). In conclusion, these results taken together suggest that the efficacy of essential oil is based on its ability to strengthen defensive factors, such as stimulant of mucus synthesis and maintains the prostaglandins content of gastric mucosa at high levels.

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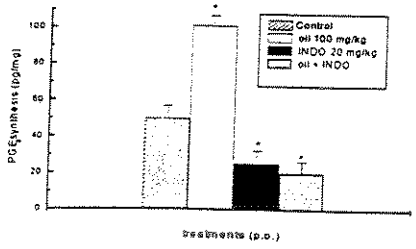
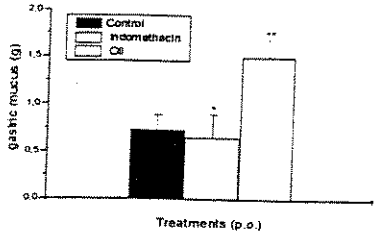
Figure 2 : Effects of oral administration of essential oil and indomethacin (INDO) on gastric PGE_2 production in rats. Each group is the mean ± SEM for 4-6 animals.



ANOVA: $F_{(4,17)} = 27.4$ $P < 0.05$, followed by Fisher's test, * $P < 0.001$

Figure 3: Effects of oral administration of essential oil and indomethacin on gastric mucus production in rats. Each group is the mean ± SEM for 4-6 animals



ANOVA: $F_{(2,17)} = 6.15$ $P < 0.05$, followed by Fisher's test, * $P < 0.05$, ** $P < 0.001$.

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IV - DISCUSSÃO GERAL

1. DHC

Vários compostos provenientes de plantas tem demostrado possuir atividade antiulcerogênica (Lewis & Hanson, 1991). As diterpenolactonas obtidas de *Croton sublyratus*, outra espécie de *Croton*, apresentaram compostos antiulcerogênicos embora com baixo rendimento e potência comparadas à DHC (Kitagawa et al., 1980).

A atividade antiucerogênica da DHC foi inicialmente avaliada em diferentes modelos de úlceras gástricas induzidas através de mecanismos diversos, tais como estresse, álcool, DAINE e um aumento da produção do suco gástrico (Desai & Parmar, 1993).

As úlceras gástricas produzidas pelo estresse por contenção e frio tem sido amplamente utilizadas para avaliar a atividade antiulcerogênica de drogas em ratos e camundongos devido à reprodutibilidade dos dados (Murakami et al., 1985) e pela semelhança com as gastrites induzidas por estresse psicológico no homem (Broddie, 1971). Neste modelo, DHC (100 mg/kg, p.o.) e cimetidina (droga-padrão, utilizada na mesma dose), protegeram significativamente a mucosa gástrica das úlceras induzidas por contenção e frio em ratos e camundongos. Os mecanismos patogênicos responsáveis pelas lesões das mucosas gástricas induzidas por estresse estão relacionados a distúrbios microcirculatórios (Koo et al., 1986), alterações na secreção gástrica, mobilidade e diminuição do muco protetor (Cho & Ogle, 1992; Cho et al., 1992).

Porém, o fator mais importante para o surgimento das úlceras por estresse é o aumento da secreção ácida gástrica caracterizado, muitas vezes,

como o fator lesivo das lesões ulcerosas (Goa & Monk 1987). A atividade anti-secretora da DHC, constatada primeiramente no modelo de ligadura do piloro em ratos, representou um importante fator para o entendimento da atividade protetora da mucosa gástrica contra as úlceras induzidas por estresse.

Outra causa comum dos danos gástricos é o excesso no consumo de álcool (Mincis et al., 1995). As lesões gástricas induzidas por etanol são causadas pela ação direta do etanol na mucosa e a acidez gástrica tem pequena contribuição na formação das lesões (Szabo, 1987). Além disso, é bem estabelecido que as úlceras induzidas por etanol não são inibidas por drogas anti-secretoras como a cimetidina, mas são inibidas por agentes que aumentam os fatores de proteção da mucosa (Robert et al.,1979). A DHC e o omeprazol (droga-padrão) foram capazes de inibir o efeito do etanol indicando, portanto, que a DHC também estaria envolvida no aumento dos fatores de proteção da mucosa, tais como muco e PG's.

Houve então, interesse em investigar se os efeitos da DHC eram dose-dependentes. Numa avaliação da curva dose-efeito de DHC no modelo de lesão gástricas induzidas por HCl/etanol em camundongos, observou-se que o índice de lesão ulcerativa (ILU) foi significativamente menor nos grupos tratados com 100 e 200 mg/kg de DHC, quando comparado aos valores de ILU obtidos para os animais controle. A DHC, na dose de 100 mg/kg p.o., apresentou um efeito protetor de cerca de 50% neste modelo farmacológico. Foi interessante a observação de que não existiu nenhuma diferença significativa entre os grupos tratados com DHC nas doses de 100 e 200 mg/kg, nem entre os animais do grupo

tratados com a dose de 50 mg/kg. Deste modo, foi evidenciado que a dose oral ótima de ação da DHC estava mesmo em torno de 100 mg/kg.

Por outro lado, a atividade citoprotetora de algumas drogas antiulcerogênicas, pela ação de agentes irritantes moderados, como é o caso do HCl/etanol nas concentrações utilizadas, tem sido objeto de diversos estudos e tem sido observado a importância da ação de PG's endógenas sobre a manutenção da integridade da mucosa gástrica na presença de diferentes agentes lesivos (Balaa & Turnage, 1990; Ligumsky et al., 1995; Ko & Cho, 1996; Tabata et al., 1996). A potente ação do lanzoprazol, neste modelo, seria devida não somente à forte inibição da secreção ácida gástrica, mas também ao fato de que os inibidores da bomba protônica do tipo do lanzoprazol possuem uma propriedade citoprotetora adicional (Yamaguchi et al., 1993). Assim, os resultados obtidos com a DHC neste experimento apontavam para ambas ações, antisecretora e citoprotetora.

Inúmeros estudos tem relatado a ação da indometacina como um potente inibidor da biossíntese de PG's (Robert, 1977; Soll et al., 1989) e existem evidências de que o aumento de certas PG's endógenas podem acentuar a resistência da mucosa gástrica contra agentes ulcerogênicos (Wallace & Granger, 1996). Verificou-se que, ao contrário da cimetidina (100 mg/kg, v.o.), a DHC administrada na mesma dose e pela mesma via, não foi capaz de promover redução significativa das lesões na mucosa gástrica induzidas pela indometacina. Este resultado apontou para uma possível inibição dos efeitos citoprotetores da DHC pelo antiinflamatório empregado, a indometacina.

Desde que uma redução na quantidade de muco da parede havia sido constatada em animais submetidos a estresse, foram estudados os efeitos da DHC e da carbenoxolona (uma droga-padrão com ação clássica neste modelo), sobre a produção de muco em lesões gástricas induzidas pelo estresse causado por contenção e frio. A DHC, ao contrário da carbenoxolona, não produziu alterações significativas na produção de muco em ratos. Portanto, foi levantada a hipótese de que os efeitos citoprotetores da DHC sobre as úlceras induzidas por etanol e estresse não seriam exercidos por um aumento da síntese e/ou retenção de muco, o que incrementaria a barreira gástrica.

Adicionalmente, está bem descrito na literatura que antioxidantes podem prevenir o surgimento de lesões gástricas tais como aquelas induzidas por etanol (Ligumnsky et al., 1995). Vários compostos antiulcerogênicos que ocorrem em plantas, tais como flavonóides, são antioxidantes e, portanto, podem apresentar-se como "scavengers" de ânions superóxido e outras espécies de oxigênio, os quais tem sido demonstrados como agentes lesivos cuja participação é importante no processo inflamatório gástrico (Lewis & Hanson, 1991; Das Banerjee, 1993).

Assim, foi investigada a possibilidade da DHC em remover radicais livres em mitocôndrias isoladas de fígado de rato. A ausência de efeitos significativos em ensaios de peroxidação lipídica mitocondrial em fígado de rato sugeriu que o mecanismo antiulcerogênico da DHC também não seria devido à proteção contra a formação de radicais livres na mucosa gástrica.

Todos os resultados obtidos até então com a DHC sugeriam que sua ação poderia ocorrer por inibição da secreção gástrica, mas a participação das

PG's no mecanismo de proteção da mucosa não havia sido totalmente descartada.

Optou-se, então, primeiramente pela completa caracterização do efeito antisecretor da DHC. Para avaliar outros possíveis mecanismos envolvidos na atividade antisecretora da DHC observou-se que na dose de 100 mg/kg, adicionada ao suco gástrico *in vitro*, não houve redução da acidez após incubação. Este resultado, portanto, indicou que a DHC não estava envolvida com um processo de simples neutralização da secreção ácida no estômago (Tan et al., 1996).

Ao contrário da cimetidina, a DHC (100 mg/kg, v.o.) também não promoveu alterações significativas nos parâmetros bioquímicos do suco gástrico quando seus efeitos foram avaliados pelo método de Shay (1945). Entretanto, numa etapa subsequente, os mesmos parâmetros bioquímicos foram avaliados em camundongos submetidos à ligadura do piloro (método de Shay modificado). Neste experimento, os animais foram pré-tratados por via intraduodenal com DHC ou cimetidina nas mesmas doses usadas para ratos. Ambas provocaram uma redução marcante na concentração total de ácidos do suco gástrico. Embora um aumento do volume gástrico total tenha sido observado após o tratamento com ambas as drogas, apenas o aumento obtido com a cimetidina foi estatisticamente significativo. Estes resultados sugeriram portanto, que a ação antiulcerogênica da DHC ligada à inibição da secreção ácida gástrica não era devida à uma ação local; mas envolvia absorção da substância e, consequentemente, uma atuação sistêmica.

Dando continuidade à investigação do efeito antiulcerogênico ligado à inibição da secreção ácida passou-se à investigação dos efeitos da DHC sobre os mediadores envolvidos no processo secretório. Para tanto, foi investigado se o efeito antiulcerogênico da DHC era decorrente do antagonismo aos receptores H_2 e/ou M_1 envolvidos com a secreção ácida gástrica. Uma série de estudos funcionais foi iniciada então e os efeitos da DHC sobre estes receptores foram investigados utilizando-se preparações isoladas. A quantificação do efeito da DHC sobre os receptores H_2 foi feita, utilizando os batimentos espontâneos do átrio direito de cobaia como modelo para o estudo da resposta mediada por estes receptores e a histamina como agonista (Spadari, 1985). Esta preparação foi escolhida por ser dificilmente dessensibilizada durante o experimento e, além disso, permitir uma avaliação de reversibilidade da interação antagonista-receptor, por um longo período de tempo (Krielaart et al., 1990).

Concentrações crescentes de DHC, adicionadas antes da realização das curvas dose-efeito cumulativas à histamina em átrio, produziram deslocamentos dose-dependentes, à direita, destas curvas. Porém, o deslocamento produzido pela menor concentração (3 μM) de DHC não foi significativo. A DHC, nas concentrações de 10 μM e 30 μM, também produziu diminuições significativas na resposta máxima da preparação; a maior dose diminuiu a freqüência cardíaca inicial. Todas as concentrações de DHC diminuíram significativamente os valores de pD_2 para histamina.

Para determinar se este efeito era específico para os receptores H_2, foi desenvolvido o mesmo experimento usando a isoprenalina como agonista dos β-adrenoceptores. Só a maior a concentração (30 μM) de DHC foi utilizada. A curva

dose-resposta à isoprenalina, como àquelas obtidas para a histamina, foi deslocada para a direita após pré-incubação com a DHC. Além disso, a resposta máxima e a freqüência inicial foram significativamente reduzidas pela DHC; entretanto, diferentemente dos resultados obtidos para a histamina, os valores de pD_2 permaneceram inalterados.

O papel da histamina sobre a secreção ácida gástrica é bem conhecido (Hirschowitz et al., 1995). No entanto, poucas vezes a secreção gástrica foi relacionada, na literatura, aos receptores β. Foi relatado que os agonistas β-adrenérgicos estimulariam a liberação ácida em estômago isolado de rato e que esta ação poderia ser antagonizada pelos bloqueadores-β (Canfield et al., 1981). Estas observações sugeriram que o bloqueio dos β-adrenoceptores no estômago, intensificaria a barreira mucosa como também poderia atenuar a ação lesiva do ácido e talvez da pepsina sobre a mucosa gástrica (Kaan & Cho, 1997). As curvas dose-resposta obtidas com a DHC para o efeito cronotrópico da histamina e isoprenalina, demostraram que existe algum tipo de antagonismo não-competitivo entre a DHC e os receptores H_2 e β em átrio direito de cobaio.

O mesmo raciocínio foi usado para analisar os efeitos da DHC sobre os receptores M_1 em fundo de estômago isolado de rato. Quando concentrações crescentes (3 e 30 μM) de DHC foram adicionadas antes da realização das curvas dose-resposta cumulativas ao carbacol, observou-se novamente um deslocamento à direita da curva ao agonista. Apesar da nítida diminuição na resposta máxima, esta redução só foi estatisticamente significativa para a dose de 30 μM. Além disso, os valores de pD_2 foram estatisticamente diferentes apenas para a maior concentração de DHC.

Estes estudos funcionais sobre os receptores farmacológicos revelaram que existe um mesmo padrão de interação entre a DHC e os receptores H_2 e β do átrio de cobaio e os receptores M_1 do fundo do estômago de rato. Embora a natureza destes antagonismos não tenha sido completamente elucidada seria possível atribuir, pelo menos em parte, algum papel a ele no efeito antisecretor da DHC.

Terminada a caracterização do efeito relacionado à inibição da secreção ácida gástrica, passou-se a fase seguinte: à avaliação dos efeitos protetores da DHC sobre a mucosa. Dentre os fatores que contribuem para a ação protetora estão as PG's que produzem diversos efeitos como a estimulação da secreção de fosfolipídeos, muco e bicarbonato; manutenção do fluxo sangüíneo gástrico durante a exposição a um agente irritante, e inibição da liberação dos mediadores inflamatórios de mastócitos e da produção de radicais livres (Motilva et al., 1996). Foi anteriormente relatado que a DHC não é capaz de aumentar a produção e/ou liberação de muco (Souza Brito, et al. 1998), tão pouco foi capaz de remover os radicais livres das mitocôndrias do fígado de rato (Rodriguez et al., 1998). Portanto, uma outra possibilidade de mecanismo de ação que intensificaria os fatores de proteção da mucosa, seria um efeito da DHC sobre a produção e a liberação de PGE_2 da mucosa. Os resultados obtidos à partir de células mucosas do estômago de ratos demonstraram que a DHC apresentou uma atividade citoprotetora evidenciada pelo aumento na produção de PGE_2 por estas células; por outro lado verificou-se ainda que a administração de indometacina (s.c.) promoveu uma significativa diminuição. Um resultado importante também foi obtido quando os ratos foram tratados com uma combinação das duas drogas, ou

seja, DHC e indometacina. A evidente redução de PGE_2, obtida pelo tratamento com as duas drogas, indicou uma nítida inibição pela indometacina do efeito citoprotetor da DHC.

Assim, foi possível obter evidências de que, além de um efeito antisecretório importante, as ações antiulcerogênicas da DHC, apresentam um componente relacionado à uma atividade citoprotetora.

Finalmente, já foi determinado que drogas antisecretoras tais como os antagonistas H_2 e/ou muscarínicos, e os agentes inibidores da bomba protônica, são capazes de inibir as úlceras de Shay (Yamaguchi et al., 1993). Como, até o momento, só haviam sido realizados ensaios para caracterização da atividade citoprotetora ou antisecretora do composto em modelos preventivos da formação de lesões gástricas, optou-se por avaliar também a propriedade curativa e cicatrizante da DHC frente a úlceras já estabelecidas, ou seja, pré-induzidas por ácido acético em ratos.

Em geral, os estudos experimentais de úlceras gástricas ou de secreção ácida falham na visão unilateral do problema e na dificuldade de se extrapolar os resultados para a espécie humana. Os animais experimentais geralmente não produzem úlceras espontaneamente e mesmo aquelas produzidas artificialmente possuem distintas diferenças com a enfermidade ulcerosa no homem (Montfort et al, 1973). Dentre os modelos de úlceras crônicas o que mais se assemelha clinicamente à úlcera humana é o de úlceras gástricas crônicas induzidas por ácido acético, denominadas também de úlceras intratáveis por sua persistência por mais de 150 dias sem a utilização de procedimentos adicionais (Takegi et al.,1969).

Na avaliação da capacidade da droga em promover a cicatrização das lesões gástricas pré-estabelecidas por ácido acético em ratos, a administração de DHC por 14 dias consecutivos (100 mg/kg/dia) falhou em reduzir estas lesões. A cimetidina, usada como droga de referência para este modelo, promoveu uma significativa cicatrização das úlceras (32%). Estes resultados demonstraram que, apesar da DHC possuir atividade antisecretória e citoprotetora em modelos animais agudos, estas atividades não foram suficientes para promover o efeito curativo necessário, ou seja, reparar o tecido previamente lesado.

Por último, no intuito de avaliar farmacologicamente a indicação popular da *C. cajucara* foram realizados também ensaios para a avaliação da toxicidade aguda tanto da DHC (sesquiterpenolactona) quanto do óleo essencial obtidos de suas cascas.

A DHC, em ensaios de toxicidade aguda em camundongos, não apresentou atividade tóxica significativa. A DL_{50} oral (878 mg/kg) foi cerca de oito vezes maior do que a dose na qual foi detectada a atividade antiulcerogênica (100 mg/kg). Portanto, este resultado indicou que a administração oral de DHC, como potencial droga antiulcerogênica, era aparentemente segura uma vez que a mesma não apresentou atividade tóxica. Tais constatações de baixa toxicidade também foram detectadas em ensaios *in vitro* envolvendo cultura de fibroblastos V 79 obtidos de pulmão de hamster chinês (Rodriguez et al, 1998), Porém, em ensaios de toxicidade subcrônica, quando a DHC foi administrada oralmente a ratos durante um período de 35 dias, alterações hepáticas significativas apareceram. O tratamento oral subcrônico com DHC não produziu qualquer alteração no peso corpóreo ou sobre parâmetros metabólicos tais como consumo

de água e alimento ou excreção de animais tratados. Houve, entretanto, uma significativa hipertrofia dose-dependente do fígado acompanhada por alterações histopatológicas severas, as quais forneceram evidências de toxicidade hepática induzida pelo tratamento com DHC (Rodríguez, 1997). Tais resultados confirmam as constatações obtidas por Nunes (comunicação pessoal) acerca da incidência, cada vez maiores, de distúrbios hepáticos, principalmente hepatite, em indivíduos que fazem uso prolongado do chá de sacaca.

2. Óleo Essencial

No intuito de estabelecer um perfil de outros componentes que pudessem estar contribuindo com atividade antiulcerogênica das cascas de *C. cajucara,* uma planta com forte indicação popular na terapêutica antiúlcera, foi avaliada também a propriedade antiulcerogênica do óleo essencial.

Na preparação popularmente utilizada, que é a infusão das cascas, existe uma perda considerável de componentes do óleo essencial por evaporação; mas fica evidente que o infuso possui óleo essencial, devido ao odor característico. Numa análise cromatográfica prévia do infuso liofilizado a 5%, foi constatada a presença do óleo essencial e a sua composição foi estudada (Nunes et al., 1998). Além disso, não foram encontrados traços de DHC no óleo extraído das cascas.

Em um estudo de toxicidade aguda em camundongos, o óleo essencial de sacaca não apresentou atividade tóxica. A DL_{50} oral (> 5 g/kg) obtida foi cerca de 90 vezes maior do que a dose utilizada para caracterizar a atividade antiulcerogênica (100 mg/kg). Porém estudos de toxicidade subcrônica serão necessários para uma melhor caracterização da toxicidade deste composto.

O estudo da atividade antiulcerogênica também foi desenvolvido utilizando-se diferentes modelos experimentais de úlceras gástricas que operam por mecanismos diversos (Desai & Parmar 1994). O efeito preventivo do óleo essencial das cascas do óleo de *C. cajucara* sobre as úlceras gástricas induzidas por diversos agentes lesivos foi avaliado tanto em ratos, quanto em camundongos.

O índice ulcerativo estabelecido foi significativamente menor nos animais tratados previamente com óleo essencial em camundongos (100 e 200 mg/kg) comparadas com o controle, nas lesões induzidas por HCl/etanol. O óleo essencial, na dose de 100 mg/kg p.o., apresentou um índice de proteção de 50% neste modelo farmacológico. Além disso, nenhuma diferença estatisticamente significativa foi observada entre os grupos tratados com o óleo nas doses de 100 mg/kg e 200 mg/kg; não houve diferença ainda entre os grupos tratados com o óleo essencial na dose de 50 mg/kg e aqueles animais do grupo controle tratados com o veículo.

O óleo essencial (100 mg/kg) também protegeu significativamente a mucosa gástrica contra as lesões ulcerosas induzidas pelo estresse por restrição e frio em camundongos em doses comparáveis à da cimetidina.

O tratamento com etanol tem a propriedade de solubilizar os constituintes do muco do estômago com uma queda concomitante na diferença de potencial transmucoso, e aumento do fluxo de Na^+ e K^+ para o lúmen, secreção de pepsina, perda dos íons H^+ e do conteúdo de histamina no lúmen (Kalia et al.,1997). Esta droga também diminui os níveis teciduais de DNA, RNA e proteínas, levando a uma estase do fluxo sangüíneo na área injuriada (Szabo 1987). Além disso, é

conhecido que as úlceras induzidas por etanol não são inibidas por agentes antisecretores tais como à cimetidina, mas sim por agentes que aumentam os fatores de defesa da mucosa como a PGE_2 (Robert et al.,1979). O óleo essencial de sacaca, na dose de 100 mg/kg, protegeu significativamente a mucosa gástrica contra injúrias produzidas pelo etanol. Estes resultados indicam que, provavelmente, o óleo essencial deva atuar aumentando os fatores de proteção da mucosa gástrica tais como o muco e/ou as PG's.

Já foi bem descrito anteriormente a ação das DAINE, como a indometacina, que reduzem a atividade da ciclo-oxigenase gástrica e diminuem os níveis de PG's endógenas (Konturek et al.,1984). Estes agentes também destroem a integridade da barreira mucosa, provocando um aumento da permeabilidade da mucosa gástrica aos íons H^+ e Na^+ e uma queda na diferença do potencial transmucoso, resultando na indução de erosões e úlceras (Droy-Lefaix, 1988). Existem evidências de que o aumento de certas PG's endógenas pode aumentar a resistência da mucosa gástrica frente a agentes ulcerogênicos tais como as DAINES (Wallace & Whittle, 1985). Neste ensaio, o óleo essencial foi capaz de produzir uma redução significativa dos danos da mucosa gástrica causados pela indometacina indicando, mais uma vez, a probabilidade de um aumento local de PG's.

Numa análise bioquímica do suco gástrico obtido de animais submetidos à ligadura do piloro demonstrou-se que a cimetidina (p.o.) provocou alterações na acidez e no volume do suco gástrico, ao passo que o óleo essencial (administrado pela mesma via e na mesma dose) provocou apenas um significativo aumento do volume gástrico. A administração intraduodenal de ambas, no entanto, provocou

efeitos similares sobre todos os parâmetros analisados. Como a cimetidina, o óleo essencial foi efetivo tanto na redução da acidez gástrica, quanto no aumento do volume do suco gástrico. Assim, foi possível inferir que as ações antisecretoras do óleo seriam devidas a um efeito sistêmico, ou seja, o efeito protetor da mucosa não dependeria do contato do óleo com a mucosa gástrica.

Estes resultados indicam claramente que a administração oral do óleo essencial de *C. cajucara* produziu significativos efeitos antiulcerogênicos em modelos animais de ratos e camundongos.

Para analisar os prováveis mecanismos de ação envolvidos com a sua atividade antiulcerogênica foram avaliadas as propriedades cicatrizantes do óleo sobre as úlceras pré-estabelecidas, além de um possível efeito citoprotetor envolvendo síntese e liberação de PGE_2 da mucosa gástrica.

As úlceras produzidas por injeção de uma solução de ácido acético na parede do estômago foram caracterizadas como úlceras intratáveis basicamente por duas razões. A primeira delas é o fato das mesmas persistirem por mais de 150 dias sem qualquer procedimento adicional; segundo, à extensão das ulcerações e as hemorragias aparentes nas bordas das lesões são confirmadas, mesmo após a ocorrência do processo cicatrizante (Takegi et al.,1969; Monfort et al.,1973). O tratamento dos ratos ulcerados com o óleo essencial de *C. cajucara* na dose de 100 mg/kg/dia, durante 14 dias, foi tão efetivo (32 % de remissão) quanto a droga-padrão, cimetidina, utilizada nas mesmas dose e via.

A inibição da acidez gástrica ou pepsina não acelera a cicatrização das úlceras, ao passo que agentes que estimulam a reepitelização ou granulação do tecido do estômago exercem uma significativa aceleração na cicatrização,

principalmente na fase inicial (Halter et al.,1995). Ito et al. (1994) relatou que a cimetidina e o omeprazol promovem a aceleração das cicatrizações das úlceras crônicas em ratos pela diminuição na secreção de gastrina; Sharstein (1996) relatou um aumento do fluxo sangüíneo mucoso em torno das úlceras, em animais experimentais, sugerindo que as PG's que promovem a vasodilatação, seriam sintetizadas muito mais na região ulcerada do que em outras partes da mucosa gástrica. O aumento do suprimento sangüíneo parece refletir a ativação da reepitelização, que requer quantidades aumentada de oxigênio e glicose.

Foi avaliado também, o efeito do óleo essencial sobre a produção de muco gástrico, o qual é considerado como um dos fatores relacionados aos mecanismos de defesa da mucosa (Garner et al., 1984). Por outro lado, a farmacologia e bioquímica da carbenoxolona tem sido extensivamente investigada, bem como aquela de várias drogas derivadas de plantas; sua eficácia está baseada em sua habilidade de estimular a síntese de muco (Bolton et al.,1978; Lewis & Hanson, 1991).

Os resultados dos estudos com o óleo essencial sobre a produção de muco, confirmam a hipótese levantada anteriormente de que o óleo essencial de *C. cajucara* seria capaz de promover um aumento significativo de sua síntese, além de reter ou aumentar a PGE_2 produzida.

Whight et al. (1982) relatam que existe um aumento significativo nos níveis de PG's em pacientes com úlceras gástricas cicatrizadas pela terapia medicamentosa, quando os mesmos foram comparados aos pacientes que apresentaram ausência de cicatrização, após 113 dias de tratamento. Vários artigos sugerem que as PG's devem estar envolvidas no processo de cicatrização

e que as mesmas também seriam responsáveis pelo início do processo, o que elevaria ainda mais os níveis de PG's em torno das úlceras refletindo-se em aumento da vascularização (Holm & Jagare, 1992). A síntese de PG's gástricas tem sido responsabilizada pela modulação de inúmeros fatores, inclusive o estresse, irritação luminal através da ativação de nervos sensoriais aferentes e eferentes colinérgicos (Guth et al., 1984). Portanto, a síntese de PG's é critica para a manutenção da integridade da mucosa gástrica; porém, seus mecanismos de ação ainda não estão completamente elucidados.

O óleo essencial promoveu um aumento duas vezes maior da síntese de PGE_2 quando comparado aos níveis de PGE_2 dos animais controle que receberam apenas o veículo. Este aumento foi completamente abolido pela administração prévia de indometacina ao animal, demostrando que o aumento de PGE_2 gástrico foi promovido pela administração oral do óleo essencial de *C. cajucara*. Este tipo de técnica permitiu determinar a capacidade de amostras de tecido em gerar PGE_2 à partir de ácido aracdônico e tem mostrado uma boa correlação com as medidas de síntese de PG's pelos tecido mucoso (Curtis et al., 1995)

Portanto, a indicação popular das cascas de sacaca como agente antiulcerogênico possui fundamentos farmacológicos evidenciados para pelo menos dois tipos de compostos ativos: a DHC, uma sesquiterpenolactona como atividade citoprotetora e antisecretora, e o óleo essencial, com atividade cicatrizante e citoprotetora sobre a mucosa gástrica.

V - CONCLUSÃO

Os ensaios experimentais *in vivo* e *in vitro* com a DHC e com o óleo essencial obtidos das cascas de *Croton cajucara* Benth. demonstraram que:

- A administração aguda oral de DHC apresentou significativa atividade antiulcerogênica em modelos de úlcera gástrica, tais como etanol, HCl/etanol, estresse por contenção e frio, e ligadura do piloro em ratos e camundongos.

- Os efeitos antiulcerogênicos da DHC não estão relacionados ao aumento da síntese de muco, tão pouco à uma propriedade antioxidativa.

- A toxicidade aguda oral da DHC é baixa; entretanto, quando administrada por periodos prolongados de tempo, a DHC mostra-se hepatotóxica.

- As ações antisecretora e citoprotetora da DHC não foram capazes de promover a cicatrização das úlceras pré-estabelecidas em ratos, ou seja, a DHC apesar de apresentar efeito antiulcerogênico preventivo, não demostra efeito curativo.

- Os mecanismos antiulcerogênicos responsáveis pela ação da DHC são decorrentes, principalmente, de dois fatores: supressão da secreção ácida pelo antagonismo não-competitivo aos receptores envolvidos e proteção da mucosa gástrica dada, fundamentalmente, pelo aumento da síntese de PGE_2.

- O óleo essencial das cascas de *C. cajucara* apresenta significativa atividade antiulcerogênica em modelos experimentais de estresse, ligadura do piloro, indometacina e HCl/etanol.

- A toxicidade aguda obtida para o óleo essencial é menor que aquela obtida para a DHC.

- O óleo essencial das cascas de *C. cajucara* apresenta atividade antiulcerogênica tanto por "prevenir" o aparecimento de lesões gástricas devido à sua ação citoprotetora (aumenta a síntese de PGE_2 e a produção de muco), quanto por "cicatrizar" as úlceras pré-existentes. O efeito curativo do óleo essencial é semelhante ao obtido para a droga-padrão, cimetidina, utilizada nas mesmas dose e via.

- Portanto, o uso popular das cascas de sacaca para o tratamento de afecções gástricas pode ser devido à associação das atividades apresentadas pelos compostos, DHC e óleo essencial, ambos presentes na preparação tradicionalmente utilizada.

VI - ABSTRACT

Croton cajucara Benth. (sacaca) is a plant species growing in the Amazon region which is popularly used to treat disorders of the digestive tract. We investigated the antiulcerogenic activity and the mechanism of action of the major constituents of the bark of the plant, i.e., the essential oil and dehydrocrotonin (DHC, a sesquiterpene lactone present in large amounts in the bark). Using acute ulcer models induced by agents such as ethanol, HCl/ethanol, pylorus ligature, indomethacin, and stress in rats and mice, we observed that the essential oil of the plant and DHC significantly reduced the ulcerative lesions in the various models studied. On the basis of these results, we investigated the possible mechanisms of action involved in the antiulcerogenic action of DHC and of the essential oil. DHC did not increase mucus production by glandular cells isolated from the stomach of rats and had no antioxidant activity on mitochondria isolated from the liver of rats. On the other hand, DHC antagonized in a noncompetitive manner the H_2 receptors of histamine in the isolated guinea pig atrium and the M_1 receptors, in the isolated rat stomach. Furthermore, DHC caused an increased PGE_2 production/synthesis by the cells of the gastric mucosa of rats. DHC did not heal preestablished ulcer in rats after 14 days of treatment. The essential oil altered the acidity and volume of gastric juice when administered intraduodenally, demonstrating a systemic action, increased the production of mucus by glandular cells of the rat stomach, and increased PGE_2 production/synthesis by cells of the rat gastric mucosa. In addition, the essential oil was able to heal (a 32% cure rate) preestablished ulcers in rats after 14 days of treatment with the same potency as cimetidine. The present results indicate that the popular use of sacaca infusion containing the essential oil to treat gastrointestinal disorders is perfectly justified by the activities of its major constituents.

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VIII – APÊNDICE

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Antinociceptive effect of Neurolaena lobata

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Antinociceptive Effect in Mice of a Hydroalcoholic Extract of *Neurolaena lobata* (L.) R. Br. and its Organic Fractions

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Abstract

An infusion of the aerial parts of *Neurolaena lobata* (L.) R. Br. (Compositae–Asteraceae) is used in Caribbean folk medicine to treat several kinds of pain. In this investigation we studied the acute oral toxicity of the hydroalcoholic extract of the plant and the antinociceptive effect of the extract and of its hexane- and chloroform-partitioned fractions, given orally, in nociception and inflammatory models in mice.

No signs of toxicity were observed for oral doses up to 5000 mg kg^{-1} in mice. Morphine hydrochloride (10 mg kg^{-1}), dipyrone sodium (200 mg kg^{-1}), the hydroalcoholic extract (1000 mg kg^{-1}), and its chloroform- and hexane-partitioned fractions (100 mg kg^{-1}) significantly inhibited acetic acid-induced abdominal constriction in mice (100, 95, 47, 62 and 60% inhibition, respectively when compared with the negative control). In the hot-plate test in mice, morphine hydrochloride, the chloroform- and hexane-partitioned fractions, but not the hydroalcoholic extract, resulted in a significant latency increase in all observation times. In the acetic acid-induced abdominal constriction in mice, pretreatment of the animals with naloxone significantly reversed the analgesic effect of morphine, but not that of the hydroalcoholic extract or of its hexane- and chloroform-partitioned fractions. Finally, administration of the hexane- and chloroform-partitioned fractions (100 mg kg^{-1}) had a significant anti-oedematogenic effect on carrageenan-induced oedema in mice.

These data show that the hydroalcoholic extract of *N. lobata* and, in particular, its partitioned fractions have significant analgesic properties when assessed through these pain models. Their antinociceptive effect might be the result of interference with the inflammatory process.

The plant family Compositae consists of approximately 920 genera and more than 19 000 species widely distributed in tropical and subtropical countries (Joly 1977). *N. lobata* (L.) R. Br., previously incorrectly named *Pluchea symphytifolia* (Miller) Gillis, is a herbaceous plant of the Compositae-Asteraceae family (Khan & Jarvis 1989) which is widespread in Central America and has also been found in north-western South America, including the north of Brazil (Pasrreiter 1995). The Guatemala Caribbeans use *N. lobata* as a remedy for several diseases, including malaria (François et al 1995), stomach pains, diabetes, skin diseases (Pasrreiter 1995) and other kinds of pain (Germosén-Robineau 1995). This plant is also used by some ethnic groups in the Antilles for cancer treatment (François et al 1995; Pasrreiter 1995). People use extremely bitter-tasting decoctions of the leaves, and other preparations. The doses and the frequency of administration differ among ethnic groups (François et al 1995).

Chemical substances isolated from *N. lobata* include a thymol derivative (Bohlmann et al 1979), twelve flavonoids including one new sulphate, several cuathemone derivatives, germacranolides, terpenoids such as α-amirine, and one obscure alkaloid (Germosén-Robineau 1995). Eleven sesquiterpene lactones, among them two named neurolenin-A and nerolenin-B (Manchand & Blount 1978) have also been isolated from this species.

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Considering its use in Central American folk medicine and the large number of chemical substances isolated from *N. lobata*, it seemed appropriate to evaluate the activity of this species. In this study we have investigated the acute toxicity of the hydroalcoholic extract of *N. lobata* and the antinociceptive effect of the extract and its hexane- and chloroform-partitioned fractions against acetic acid-induced abdominal constriction and the hot-plate analgesic test. Because of the use of this species for cancer treatment (François et al 1995; Pasrreiter 1995), we also investigated the possible involvement of its antinociceptive effect with opioid receptors. Finally, the anti-oedematogenic effect of the hexane- and chloroform-partitioned fractions was investigated using carrageenan-induced paw oedema in mice.

Materials and Methods

Drugs

Dipyrone sodium (Hoechst Marion Roussel, Brazil) and the extract and its hexane- and chloroform-partitioned fractions were dissolved in 12% Tween 80 (Synth, Brazil) solution. Acetic acid, morphine hydrochloride, naloxone hydrochloride, carrageenan and indomethacin (Sigma, MO) were dissolved in 0·9% NaCl (saline) or 5% $NaHCO_3$ solution. All reagents were of a high grade of purity. Substances, reagents and extract or fractions were prepared immediately before use.

Animals

Experiments were performed, during the morning, on Swiss albino mice, 30±5 g, from the Central Animal House of the Universidade Estadual de Campinas (Cemib/Unicamp). They were fed a certified Nuvilab CR-a (Nuvital) diet, had free access to tap water and were kept in the animal house under standard conditions of 12 h light–dark, humidity, and temperature. Animals were fasted before the assays because the standard drugs or extract and fractions of *N. lobata* were always administered orally (p.o.), except for indomethacin, morphine and naloxone. All experiments were performed according to current guidelines for the care of laboratory animals and ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann 1983).

Preparation of Neurolaena lobata *extract and fractions*

The leaves of *N. lobata* were collected by a botany group from Enda-Caribe, Santo Domingo, Dominican Republic, coordinated by Dr Lionel Germonsén-Robineau. The dried leaves were minced and extracted with ethanol–water, 9:1, in a Soxhlet apparatus. The resulting extract was evaporated to dryness under vacuum at 50°C and the residue was successively submitted to hexane and chloroform partition to obtain appropriate partitioned fractions. The solvent was then removed from the resulting fractions by evaporation.

Acute toxicity and LD50

Studies of the acute toxicity of the hydroalcoholic extract were performed on mice. Increasing doses of *N. lobata* hydroalcoholic extract were administered to groups of 10 animals for each dose level after a 12-h fast. The animals were observed for 14 days, when the number of survivors was recorded. The acute toxicologic effect was estimated by the method described by Souza Brito (1995) and was expressed as LD50 (the dose resulting in the death of half the mice) according to Litchfield & Wilcoxon (1949).

Abdominal constriction response caused by intraperitoneal injection of acetic acid

The response to intraperitoneal injection of a 0.6% acetic acid solution; contraction of the abdominal muscle and stretching of the hind limbs; was induced according to procedures described by Koster et al (1959). Animals were pretreated with the hydroalcoholic extract ($1000\,mg\,kg^{-1}$), or its hexane- or chloroform-partitioned fractions ($100\,mg\,kg^{-1}$) and negative-control animals received a similar volume of 12% Tween 80 ($10\,mL\,kg^{-1}$). Positive-control mice received dipyrone ($200\,mg\,kg^{-1}$, p.o.) and subcutaneous (s.c.) morphine ($10\,mg\,kg^{-1}$). The drugs were administered 30 min before injection of 0·6% acetic acid. After challenge, pairs of mice were placed in separate transparent boxes and the number of abdominal constrictions over a period of 6–21 min were counted. Antinociceptive activity was expressed as the reduction of the number of abdominal constrictions. The number of abdominal constrictions and stretchings was recorded and percentage protection was calculated by use of the formula:

$$[(\text{control mean} - \text{treated mean})/\text{control mean}] \times 100$$

Hot-plate test

The hot-plate test was used to measure latencies according to the method described by Eddy & Leimback (1953), with minor modifications. In these experiments the hot-plate apparatus (Ugo Basile, Model-DS 37) was maintained at 56±1°C. Animals were placed in a 24-cm diameter glass cylinder on the heated surface and the time between

placement and shaking or licking of the paws or jumping was recorded as latency. Latency was recorded for control mice (treated with vehicle) and for animals pretreated with morphine (10 mg kg^{-1}, s.c.) used as positive-control or pretreated with the hydroalcoholic extract (1000 mg kg^{-1}), or with its hexane- or chloroform-partitioned fractions (100 mg kg^{-1}). All substances were administered 30 min before the beginning of the experiment. Animals were selected 24 h previously on the basis of their reactivity in the test. Only animals showing a reaction within the 3·9–6·9 s range were selected. Negative-control animals received a similar volume of 12% Tween 80 (10 mL kg^{-1}, p.o.). All animals were observed before (0 min) and 30, 60 and 90 min after drug administration. The latency period of 30 s was defined as complete analgesia.

Analysis of the mechanism of analgesic action of Neurolaena lobata *hydroalcoholic extract and its chloroform- and hexane-partitioned fractions*

The possible participation of the opioid system in the antinociceptive effect of the hydroalcoholic extract of *N. lobata* and its chloroform- and hexane-partitioned fractions was investigated. To analyse this mechanism we also used the acetic acid-induced abdominal constriction model in mice, with some modifications. Animals were pretreated with intraperitoneal (i.p.) naloxone (5 mg kg^{-1}) 15 min before oral administration of the hydroalcoholic extract (1000 mg kg^{-1}) or its chloroform- or hexane-partitioned fractions (100 mg kg^{-1}), or subcutaneous administration of morphine (10 mg kg^{-1}). Control animals received a similar volume of 12% Tween 80 (10 mL kg^{-1}) orally.

Carrageenan-induced paw oedema in mice

The method utilized was similar to that described by Henriques et al (1987) who used groups of male mice. Pretreatment with indomethacin (20 mg kg^{-1}, s.c.; used as positive control) or with its hexane- or chloroform-partitioned fractions (100 mg kg^{-1}, p.o.) was 30 min before injection of 300 μg carrageenan (1% suspension in normal saline). The paws were weighed 3 h after carrageenan injection. The increase in weight caused by the irritant was found by subtracting the weight of the untreated left paw from that of the treated right paw.

Statistical analysis

Results are expressed as means ± standard deviation (s.d.) or means ± standard error of the mean (s.e.m.); statistical significance between results from different groups was assessed by analysis of variance followed by Dunnett's pairwise test. P values $< 0·05$ were considered indicative of significance.

Results and Discussion

In this study relatively large doses of the plant extract (1000 mg kg^{-1} p.o.) were needed to elicit pharmacological action. In our toxicologic assay oral doses up to 5000 mg kg^{-1} resulted in no signs of toxicity in mice, and no significant changes in daily body weight or organ weight were observed during the next 14 days (results not shown). This might be a reflection of the low concentrations of the active and toxic substances in the extract. This confirmed the widely reported low toxicity of the plant extract. Considering the high experimental dose, this probably indicates that, at the usual doses employed by man (estimated to be about 10% of the dose used here), the plant extract would probably not have toxic effect. However, it should be stressed that extrapolations of the effects of potentially toxic substances from animals to man cannot always be made (Tanira et al 1996).

We also investigated the analgesic and anti-oedematogenic effect of the hydroalcoholic extract of *N. lobata* leaves and of its partitioned fractions in mice, by use of chemical (acetic-acid induced abdominal constriction) and thermal (hot-plate test) pain models and one acute inflammatory assay.

The abdominal constriction elicited by acetic acid has been used to assess the potential analgesic activity of drugs. Morphine, dipyrone, the hydroalcoholic extract, and its chloroform- and hexane-partitioned fractions significantly inhibited acetic acid-induced abdominal constriction in mice, by 100, 95, 47, 62 and 60%, respectively, compared with control animals (Table 1). The data show that the percentage inhibition of the abdominal

Table 1. Effect of the hydroalcoholic extract of *Neurolaena lobata*, and of its chloroform- and hexane-partitioned fractions, on acetic acid-induced abdominal constriction in mice.

Treatment	Dose (mg kg^{-1})	Number of constrictions	Inhibition (%)
Control	–	41·0 ± 3·11	–
Morphine	10	0*	100
Dipyrone	200	2·0 ± 0·58*	95·1
Hydroalcoholic extract	1000	21·7 ± 3·09*	47·1
Hexane-partitioned fraction	100	16·4 ± 2·88*	60·0
Chloroform-partitioned fraction	100	15·4 ± 1·62*	62·4

Each value is the mean ± s.d. of results from seven animals. Significantly different from the respective control value; analysis of variance $F(4,30) = 229$ ($P < 0·05$); Dunnett's test *$P < 0·001$.

constrictions afforded by 100 mg kg^{-1} of the chloroform- and hexane-partitioned fractions was equivalent to that afforded by 200 mg kg^{-1} dipyrone. These data also show the significant antinociceptive activity of these fractions, considering that the partitioned fractions obtained from the hydroalcoholic extract are only semi-purified. Dipyrone and morphine, used as positive reference controls, also had significant antinociceptive effects in this pain model.

Collier et al (1968) postulated that acetic acid acts indirectly by inducing the release of endogenous mediators that stimulate the nociceptive neurons sensitive to non-steroidal anti-inflammatory drugs, to narcotics and to other centrally acting drugs (Vaz et al 1996). Thus, the abdominal constriction elicited by acetic acid would be considered a less selective antinociceptive model.

Although the hot-plate test is commonly used to assess narcotic analgesics, other centrally acting drugs, including sedatives and muscle relaxants or psychotomimetics are active in this test (Vaz et al 1996). In the hot-plate test there was no significant difference between pretreatment latency values obtained on the day of the test (time 0) and those obtained 24 h before during animal selection (data not shown). The results presented in Table 2 show that oral administration of the hexane- and chloroform-partitioned fractions, but not of the hydroalcoholic extract, significantly increased the latency at all observation times (30, 60 and 90 min). Morphine, used as a reference drug, had the same significant antinociceptive effect at all observation times when compared with its own control values. These results show that in both experiments the active principle(s) of *N. lobata* was (were) present in the hexane- and chloroform-partitioned fractions from the leaves.

Because of the use of this plant for cancer treatment (François et al 1995; Pasrreiter 1995), we supposed possible action of the extract or its partitioned fractions on opioid receptors and used naloxone, a non-selective antagonist of the opioid receptors, in an attempt to gain some insight into the mechanisms involved in the antinociceptive properties of the hydroalcoholic extract and its hexane- and chloroform-partitioned fractions. In some animal models naloxone apparently acts by antagonizing the actions of endogenous opioids mobilized by pain or stress (Faden 1988). The data shown in Table 3 indicate that the non-selective opioid antagonist naloxone (5 mg kg^{-1}, i.p.) did not consistently reverse the extract- and the fractions-induced antinociception when assessed against acetic acid-induced pain. Naloxone significantly reversed the morphine-induced antinociceptive effect in the chemical pain model (acetic acid-induced abdominal constriction). Our results show, however, that the organic fractions obtained from *N. lobata* did not act by interaction with the opioid system.

Pretreatment with the hexane- and chloroform-partitioned fractions significantly reduced carrageenan-induced oedema, by 44 and 68%

Table 3. Effect of the hydroalcoholic extract of *Neurolaena lobata*, and of its chloroform- and hexane-partitioned fractions, on acetic acid-induced abdominal constriction in mice pretreated with intraperitoneal naloxone (5 mg kg^{-1}).

Treatment	Oral dose (mg kg^{-1})	Number of constrictions	Inhibition (%)
Control	–	28·0 ± 3·03	–
Morphine	10	0	100
Naloxone + morphine	10	25·8 ± 2·48	13·6
Naloxone + hydroalcoholic extract	1000	13·0 ± 1·67*	53·6
Naloxone + hexane-partitioned fraction	100	9·7 ± 1·03*	65·4
Naloxone + chloroform-partitioned fraction	100	8·5 ± 0·55*	69·6

Each value is the mean ± s.d. of results from eight animals. Significantly different from the respective control value; analysis of variance $F(5,42) = 225$ ($P < 0.05$); Dunnett's test *$P < 0.001$.

Table 2. Effect of the hydroalcoholic extract of *Neurolaena lobata*, and of its chloroform- and hexane-partitioned fractions, in the hot-plate test in mice.

Observation time (min)	Latency (s)				
	Control	Morphine	Hydroalcoholic extract	Hexane-partitioned fraction	Chloroform-partitioned fraction
0	6·62 ± 0·92	6·75 ± 1·04	5·87 ± 0·99	6·25 ± 1·28	6·12 ± 0·99
30	7·25 ± 1·16	15·3 ± 2·90†	7·50 ± 1·31	9·50 ± 1·07*	9·75 ± 1·04*
60	6·25 ± 1·28	16·5 ± 3·59†	9·0 ± 2·33	11·1 ± 1·81*	13·9 ± 1·89†
90	7·12 ± 0·83	13·8 ± 3·49†	7·50 ± 1·07	10·8 ± 1·60*	13·6 ± 2·07†

Each value is the mean ± s.d. of results from eight animals. Significantly different from result at 0 min for each group value; analysis of variance $F(4,35)$ 30 min = 24·6; 60 min = 22·4; 90 min = 17·9 ($P < 0.05$); Dunnett's test, *$P < 0.05$; †$P < 0.001$.

Table 4. Effect of the chloroform- and hexane-partitioned fractions of *Neurolaena lobata* on carrageenan-induced paw oedema in mice.

Treatment	Dose (mg kg^{-1})	Paw weight (mg)	Inhibition (%)
Control	–	63·7 ± 5·51	–
Indomethacin	20	23·3 ± 5·67†	63·4
Hexane-partitioned fraction	100	35·8 ± 4·89*	43·8
Chloroform-partitioned fraction	100	20·2 ± 6·18†	68·3

Each value is the mean ± s.e.m. of results from seven animals. Significantly different compared with the respective control value; analysis of variance $F(3,24) = 12·6$ ($P < 0·05$); Dunnett's test $*P < 0·05$; $†P < 0·001$.

respectively, 3 h after phlogistic compound injection. These results, reported in Table 4, show that these fractions are not endowed with morphinomimetic property; the effect might be related to anti-inflammatory action on the acute inflammatory processes.

The punctual antinociceptive mechanism of the extract and its partitioned fractions cannot be determined from these data or with these tests because they measure different types of pain, i.e. those induced by chemical- (abdominal constriction test), thermal- (hot-plate test) or inflammatory (paw oedema) agents. The mechanism underlying this analgesic effect might be related to inhibition of prostaglandin synthesis or to other endogenous mediators of the inflammatory process, such as histamine and 5-hydroxytryptamine (Emim et al 1992).

Although the chemical principles responsible for the antinociceptive effects of the fractions obtained from *N. lobata* are not known, much of the action might be related to the presence of the terpenoids, sequiterpene-lactones, flavonoids, or some still unidentified alkaloid (Germosén-Robineau 1995).

These preliminary findings lend support to the use of this plant in folk medicine for pain treatment, mainly because of its low toxicity. Further work is needed to clarify the mechanism of its antinociceptive action.

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