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Eryvelton de Souza Franco

Estudo do efeito gastroprotetor de extratos e de frações semipurificadas de *Chresta martii* (DC.) H. Rob. e identificação do seu composto majoritário

Recife
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Tese apresentada como requisito complementar para obtenção do grau de Doutor em Biotecnologia, ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal de Pernambuco na qualidade de Instituição Nucleadora da Rede Nordeste de Biotecnologia.

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"Talvez não tenha
conseguido fazer o melhor,
mas lutei para que o melhor
fosse feito. Não sou o que
deveria ser, mas Graças a
Deus, não sou o que era
antes".

(Marthin Luther King)

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ao fim de mais uma etapa
acadêmica na minha vida;

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Eneida Franco e Tia Quina Dias.

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LISTA DE ABREVIATURAS

^{13}C-RMN - Espectroscopia de Ressonância Nuclear Magnética – Carbono 13

^{1}H-RMN - Espectroscopia de Ressonância Nuclear Magnética – Hidrogênio

AINEs - Antiinflamatório Não Esteroidais

AMPc - Monofosfato Cíclico de Adenosina

ANVISA - Agência de Nacional de Vigilância Sanitária

CCD - Cromatografia em Camada Delgada

COX - Ciclooxigenase

COX-1 – Ciclooxigenase - 1

COX-2 – Ciclooxigenase - 2

CSW - Camundongos Swiss Webster

EACm - Extrato Acetato de Etila de *Chresta martii*

ECCm - Extrato Ciclohexano de *Chresta martii*

EECm - Extrato Etanólico de *Chresta martii*

EROs - Espécies Reativas de Oxigênio

GMPc - Monofosfato Cíclico de Guanosina

GSH - Glutationa Reduzida

H^+/K^+-ATPase - Bomba de Hidrogênio/Potássio

H2 - Receptor de Histamina tipo 2

HCl - Ácido Clorídrico

HL-60 - Linhagem de Leucemia Promielocítica Humana

HPLC - Cromatografia Líquida de Alta Eficiência

LS - Lactonas Sesquiterpênicas

M3 - Receptor Muscarínico tipo 3

MCF-7 - Linhagem de Câncer de Mama

NCI-H292 - Linhagem de Carcinoma de Pulmão Humano

NO - Óxido Nítrico

PGE1 - Prostaglandina E1

PGE2 - Prostaglandina E2

PGs - Prostaglandinas

pH - Potencial Hidrogênio Iônico

RDC - Reunião das Diretorias Colegiadas

RE - Resolução

Rf - Fator de Retenção

SNC - Sistema Nervoso Central

SST - Somatostatina

TGI - Trato Gastrointestinal

TNF-α - Fator de Necrose Tumoral Alfa

RESUMO

Chresta martii (Asteraceae) espécie encontrada na região do Xingó (semiárido do nordeste brasileiro), utilizada localmente no tratamento de disfunções gástricas. O presente trabalho teve como objetivo avaliar a toxicidade aguda e genotóxicidade (*in vivo*), a citotóxicidade (*in vitro*) e atividade gastroprotetora de extratos e de frações semipurificas de *Chresta martii* e identificar seu composto majoritário. Três extratos orgânicos foram obtidos a partir das partes aéreas secas de *C. martii* utilizando como solvente (ciclohexano - ECCm, acetato de etila - EACm e etanol - EECm); o EACm foi fracionado em coluna de sílica gel 60 eluido com clorofórmio [F1; rendimento (10%)], clorofórmio/ acetato de etila (1/1); [F2; rendimento 6%)], acetato de etila [F3; rendimento (8%)] e acetato de etila/metanol (1/1) [F4; rendimento (5%)]. A caracterização fitoquímica dos extratos foi determinada por HPLC, enquanto as frações semipurificadas foram avaliadas por CCD e os compostos isolados oriundos do refracionamento foram identificados por Espectroscopia de Ressonância Nuclear Magnética ^{1}H-RMN e ^{13}C-RMN. Os extratos foram avaliados quanto a toxicidade aguda em camundongos Swiss webster (CSW) (2000 – 50 mg/kg; *i.p.* ou *v.o.*), citotoxicidade *in vitro* (50 µg/mL) frente à linhagens de células cancerígena (HL-60, NCI-H292 e MCF-7) humana e genotoxicidade em CSW (50 mg/kg; *i.p.*), através da técnica de micronúcleo em células de medula óssea. Os três extratos foram avaliados quanto a atividade gastroprotetora (50, 100 ou 200 mg/kg; *v.o.*) frente a lesões gástricas induzidas por indometacina (40 mg/kg, *s.c.*) ou etanol (0,2 mL/animal; *v.o.*) em CSW machos (25–30 g). As frações semipurificadas F1, F2, F3, F4 (50 mg/kg; *v.o.*) ou F1 (12,5, 25 ou 50 mg/kg; *v.o.*) foram avaliadas quanto a gastroproteção frente ao modelo de úlcera induzida por etanol. Os grupos controles positivos foram tratados com ranitidina (80 mg/kg, *v.o.*) ou omeprazol (30 mg/kg; *v.o.*) ou salina 0,9% (5 mL/kg; *v.o.*) controle negativo. O ECCm (2000 mg/kg; *v.o.* ou *i.p.*) não apresentou nenhum indício de toxicidade aguda ou registro de óbito. A DL_{50} estimada para (EACm e EECm) foi de 500 mg/kg; *v.o.* e 200 mg/kg; *i.p.*. O EACm (50 µg/mL) inibiu o crescimento das células tumorais HL60 (96,54% ± 0,22%), NCIH292 (73,43% ± 1,07%) e MCF-7 (15% ± 3,59%). A fração F1 foi capaz de induzir a formação de micronúcleo nos eritrócitos policromáticos (66,67% ± 4,32%) de CSW. Dentre os extratos avaliados, o EACm exibiu significante ($p<0.05$) atividade gastroprotetora nos modelos utilizados. A F1 (25 mg/kg; *v.o.*) revelou atividade gastroprotetora superior ($p<0.05$) aquela exibida pela

ranitidina (80 mg/kg; *v.o.*) no modelo de úlcera induzida por etanol. O refracionamento da F1 originou 23 subfrações e dessas foram obtidos, por recristalização, dois compostos de cor amarela, amorfo, Rf: 0,46 e 0,31 (acetato de etila: clorofórmio 5:5). Os composto isolados foram identificados como flavonas: Chrisoeriol (rendimento – 0,43%) e o 3',4'-Dimetoxiluteolina (rendimento – 0,58%). Os extratos (EACm e EECm) e a fração (F1) de *Chresta martii* apresentaram potencial citotóxico (*in vitro*) e genotóxico (*in vivo*), além de exibir toxicidade aguda classificada como de leve a moderada. A identifição do composto majoritário (3',4'-Dimetoxiluteolina) presente na *Chresta martii* fornece provável suporte racional para a propalada utilização da espécie no tratamento de distúrbios gastrointestinal, conforme informações etnofarmacológica e experimentais em camundongos.

Palavras-chave: *Chresta martii*. Asteraceae. 3',4'-Dimetoxiluteolina. Doenças gástricas. Úlcera gástrica. Camundongos. Flavonas

ABSTRACT

Chresta martii (Asteraceae), found in the Xingó region (semi-arid region in the Northeast of Brazil), is locally used for treating gastric disorders. This study aimed to evaluate the acute toxicity and genotoxicity (*in vivo*), cytotoxicity (*in vitro*) and gastroprotective activity of crude extracts and semi-purified fractions of *Chresta martii*, as well as, identify its major compound. Three organic extracts were obtained from the dried aerial parts of *C. martii* (cyclohexane - ECCM, ethyl acetate – EACm, ethanol - EECm); The EACm was fractionated on a silica gel 60 column eluted with chloroform [F1; yield (10%)] chloroform / ethyl acetate (1/1); [F2; yield 6%)] ethyl acetate [F3; yield (8%)] and ethyl acetate / methanol (1/1) [F4; yield (5%)]. The phytochemical characterization of the extracts was determined by HPLC, while the semi-purified fractions were analyzed by TLC, and the isolates compounds derived from the subdivision were identified by Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR and ^{13}C-NMR). The extracts were evaluated for acute toxicity in male Swiss mice (2000-50 mg/Kg; *i.p.* or *p.o.*), *in vitro* cytotoxicity (50 mg/mL) through the cancer cell lines (HL-60, NCI-H292 and MCF-7) and human genotoxicity in mice (50 mg/Kg, *i.p.*) by micronucleus technique in bone marrow cells. The three extracts were evaluated for gastroprotective activity (50, 100 or 200 mg/Kg; *p.o.*) against gastric lesions induced by indomethacin (40 mg/Kg, *s.c.*) or ethanol (0.2 ml/animal, *p.o.*) in male mice (25-30 g). The semi-purified fractions F1, F2, F3, F4 (50 mg/Kg, *p.o.*) or F1 (12.5, 25 or 50 mg/Kg, *p.o.*) were evaluated for gastroprotective activity through ulcer model induced by ethanol. The positive control groups were treated with ranitidine (80 mg/Kg, *p.o.*) or omeprazole (30 mg/Kg, *p.o.*) and negative control with saline (5 ml/Kg, *p.o.*). ECCm (2000 mg/Kg; *p.o.* or *i.p.*) did not show any evidence of acute toxicity or death record. The estimated LD_{50} (EACm and EECm) was 500 mg/Kg; *p.o.* and 200 mg/Kg; *i.p*. The EACm (50 mg/mL) inhibited the growth of tumor cells HL-60 (96.54% ± 0.22%), NCI-H292 (73.43% ± 1.07%) and MCF-7 (15% ± 3.59%). The F1 fraction was able to induce the formation of micronucleus in polychromatic erythrocytes (66.67% ± 4.32%) of mice. Among the extracts evaluated, the EACm showed a significant ($p<0.05$) gastroprotective activity in the studied models. The F1 fraction (25 mg/Kg; *p.o.*) showed a higher gastroprotective activity ($p <0.05$) to that exhibited by ranitidine (80 mg/Kg; *p.o.*) in ulcer model induced by ethanol. The fractionation of F1 fraction, originated 23 subfractions and these were obtained, by recrystallization, two compounds

with yellow color and amorphous, Rf: 0.46 and 0.31 (ethyl acetate: chloroform 5:5). The isolated compounds was identified as flavones: Chrisoeriol (yield - 0.43%) and 3 ', 4'-Dimetoxiluteolina (yield - 0.58%). The extracts (EACm and EECm) and the fraction (F1) of *Chresta martii* showed cytotoxic (*in vitro*) and genotoxic potential (*in vivo*), besides presented an acute toxicity classified as mild to moderate. The identification of the major compound (3',4'-Dimetoxiluteolina) contained in *Chresta martii* likely provides reasonable support for the widespread use of this plant in the treatment of gastrointestinal disorders, as observed trough the ethnopharmacological and experimental data obtained in mice.

Keywords: *Chresta martii*. Asteraceae. 3',4'-dimethoxyluteolin. Gastric diseases. Gastric ulcer. Mice. Flavones

SUMÁRIO

1 INTRODUÇÃO

As informações oriundas de plantas medicinais com propósito terapêutico foram acumuladas durante séculos, e muitas dessas empíricamente. O conhecimento sobre plantas medicinais representou e ainda representa o único recurso terapêutico de muitas comunidades e grupos étnicos (DI-STASI, 1996; NOLLA, 2005; ZAGO *et al*., 2009).

Foi a partir do final do século XX que a prática da Fitoterapia tornou-se difundida por todo mundo, sendo esta ciência definida como a terapêutica que utiliza os medicamentos cujos constituintes ativos são plantas ou derivados de espécies vegetais, e que tem sua origem respaldada no conhecimento e no uso popular (BRASIL, 2012).

Vários estudos têm testado a aplicação terapêutica e toxicidade de diversos produtos derivados de plantas medicinais. Desta forma, a utilização de uma diversificada flora tem sido validada, ainda que em ensaios pré-clínicos no tratamento de doenças do trato gastrointestinal (BACCHI, 1986; REPETTO; LLESUY, 2002; ABDULLA *et al.,* 2010; AL-ATTAR, 2011), entre outras patologias.

Contudo, o desenvolvimento tecnológico voltado à área da química orgânica possibilitou ao homem ir mais longe no que concerne a identificação de ativos reponsáveis por diferentes efeitos farmacológicos, atribuídos às espécies vegetais, não se contentando em apenas comprovar a atividade dos extratos brutos como outrora por muitos pesquisadores foram relatados. Na atualidade, os estudos farmacológicos e químicos cada vez mais são direcionados com o propósito de desvendar mecanismos de ação e identificar seus ativos reponsáveis. Muitas vezes, os resultados dessas buscas tornam-se atraentes à indústria farmacêutica, pois essa pode utilizar os isolados das plantas medicinais como parâmetro para padronização de fitoterápico ou mesmo como protótipo para a síntese ou semi-síntese de moléculas farmacologicamente ativas. Esse cenário encontra respaldo nos estudos que avaliam a atividade terapêutica e a toxicidade de diversos produtos derivados de plantas medicinais.

Neste âmbito, as espécies da família Asteraceae têm demonstrado significativa atividade analgésica, anti-inflamatória e antiulcerogênica (PEREIRA *et al.,* 2005; SILVÉRIO *et al.*, 2008; DIAS *et al.,* 2009). Especificamente muitas espécies dessa família têm apresentado atividade antiulcerogênica em diferentes modelos experimentais, como por exemplo: *Achillea millefolium* (BAGGIO *et al.,* 2003), *Mikania laevigata* (BIGHETTI, 2005), *Senecio brasiliensis* (TOMA *et al.,* 2004),

Franseria artemisioides, *Bacharis genistelloides*, *Bacharis rubricaulis*, *Bacharis illinita* (GONZALES, 2000; VERDI, 2005).

Quanto à prospecção fitoquímica tem-se verificado na família Asteraceae a presença de sesquiterpenoides, poliacetilenos, diterpenoides, triterpenoides, flavonóides, cumarinas, benzofuranos e benzopiranos (EMERENCIANO, 1998, SILVA *et al.*, 2012; SILVA *et al.,* 2013). Entretanto, a identificação de composto ativo isolado responsável pelo efeito antiulcerogênico, por exemplo, e o mecanismo de ação por ele desencadeado muitas das vezes não são completamente conhecidos.

A espécie *Chresta martii* (DC.) H. Rob. pertencente à família Asteraceae, com características de Caatinga é encontrada entre rochas às margens do rio São Francisco, principalmente na região do Xingó no estado de Sergipe localizada no Nordeste brasileiro. Ela é amplamente utilizada pela população local no combate as doenças do trato gastrintestinal (ALMEIDA *et al.,* 2005; ALMEIDA *et al.,* 2006; ALBUQUERQUE *et al.,* 2007). Entretanto, poucos são os estudos pré-clínicos com o intuito de avaliar a eficácia e segurança de extratos e frações semipurificadas de *C. martii*. Nesse contexto, o estudo atual contribuirá para avaliar a segurança, eficácia e identificar o composto majoritário presente nas partes aéreas de *C. martii* com provável atividade gastroprotetora.

2 REVISÃO DE LITERATURA

2.1 SISTEMA GÁSTRICO

2.1.1 Fisiologia das secreções gástricas

A secreção de ácido gástrico é um processo contínuo e complexo, no qual múltiplos fatores centrais e periféricos contribuem para uma meta comum: secreção de H^+ pelas células parietais. Os fatores neuronais (acetilcolina), parácrinos (histamina) e endócrinos (gastrina) regulam a secreção de ácido, através da ativação de receptores específicos (M3, H2 e CCK2, respectivamente) que se localizam na membrana basolateral das células parietais no corpo e fundo gástricos. Nessas células (parietais) o AMP cíclico e as vias dependentes de Ca^{2+} ativam a H^+/K^+-ATPase (a bomba de prótons), que efetua a troca de íons hidrogênio e potássio através da membrana celular parietal. Essa bomba gera o maior gradiente iônico conhecido entre os vertebrados, com um pH intracelular de cerca de 7,3 e um pH intracanalicular de cerca de 0,8 (BRUNTON *et al.,* 2007).

A secreção endócrina (gastrina) é o indutor mais potente para secreção de ácido, sendo está liberada através de múltiplas vias de estimulação, incluindo ativação do SNC, distensão local e componentes químicos do conteúdo gástrico. A gastrina estimula a secreção ácida indiretamente ao induzir a liberação de histamina pelas células enterocromafins; um efeito direto sobre as células parietais também desempenha um papel menos importante (BRUNTON *et al.,* 2007).

A somatostatina (SST) inibe a secreção de ácido gástrico. A acidificação do pH luminal gástrico para valores menores que três estimula a liberação de SST que, por sua vez, suprime a produção de gastrina em uma alça de retroalimentação negativa. As células produtoras de SST estão diminuídas em pacientes, por exemplo, com infecção por *Helicobacter pylori*, e a consequente redução do efeito inibitório da SST podem contribuir para produção excessiva de gastrina (SCHUBERT, 2009).

Contudo, sabe-se que a hipersecreção gástrica também pode estar associada a Síndrome de Zollinger-Ellison, hiperplasia das células-G, aumento na quantidade de células parietais e a ausência de equilíbrio fisiológico entre hormônios gástricos antagonistas, gastrina e somatostatina; entretanto, a estimulação da secreção de ácido clorídrico pode estar relacionada com hipersensibilidade colinérgica e ação

parassimpática, funcionando como co-fator em danos erosivos na mucosa gástrica (YUAN *et al.*, 2006).

2.1.2 Distúrbios do sistema gástrico

Nos últimos 20 anos o estudo das doenças relacionadas à úlcera gástrica e duodenal, tem aumentado significativamente devido à identificação de várias técnicas, as quais têm possibilitado a avaliação mais detalhada da mucosa gástrica (BRZOZOWSKI *et al.,* 2005). Na média populacional o risco de complicação com úlcera aumenta quatro vezes, resultando em 1,25 hospitalizações adicionais para cada 100 pacientes por ano (HAWKEY, 2000).

As doenças ulcerativas do trato gastrointestinal dependem de duas condições: presença de ácido e predisposição das mucosas às lesões por fatores diversos. Não há maneiras estabelecidas de interferir farmacologicamente com as predisposições, genéticas ou não, da mucosa aos danos (BRZOZOWSKI *et al.*, 2005).

As úlceras ocorrem frequentemente no duodeno (úlcera duodenal), onde mais de 95% estão localizadas na sua primeira porção, e 90% próximo à junção do piloro com a mucosa duodenal. No estômago (úlcera gástrica), as úlceras se localizam mais comumente no antro (60%) e na junção do antro com o corpo, na pequena curvatura (25%). A incidência de úlceras gástricas parece ser ligeiramente maior em homens em relação às mulheres (1,3: 1), sendo que a faixa etária de maior ocorrência das úlceras duodenais é de 30-55 anos, e das úlceras gástricas é de 50-70 anos (ABITBOL, 2012).

Fatores como fumo, álcool, estresse, uso de medicamentos, faixa etária, infecção pela *Helicobacter pylori*, hereditariedade de afecções pépticas, entre outros, podem desempenhar diferentes papéis na gênese da doença (SAUL, 2007). A patogenia da doença ulcerosa péptica é mais bem representada como um complexo cenário envolvendo o desequilíbrio entre os fatores de defesa da mucosa (bicarbonato, muco, prostaglandinas, fluxo sanguíneo, óxido nítrico, fatores de crescimento, etc.) e fatores agressivos que compreendem os agentes químicos, que podem ser endógenos (HCl, pepsina) ou exógenos (etanol, antiinflamatórios não esteroidais), e agentes biológicos (*Helicobacter pylori*) (NATALE *et al.*, 2004) (Fig.1).

Dessa forma, a profilaxia atrelada ou não a terapias medicamentosas preventivas, de maneira a intensificar os mecanismos de defesa fisiológicos, continuam sendo o meio mais utilizado de controle da secreção ácida, pois revertem os danos

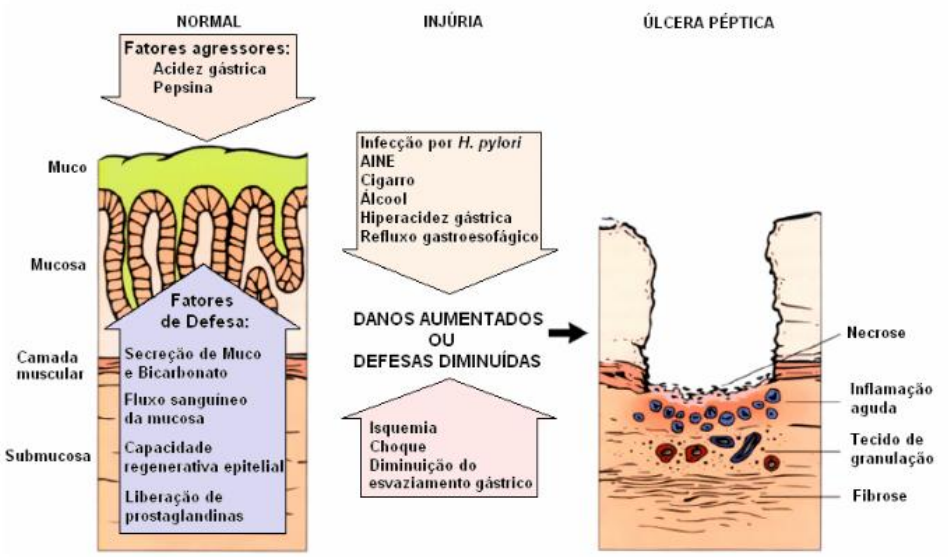
causados à mucosa e controlam os eventos inflamatórios que se sucedem após a instalação das lesões as quais são tarefas bem mais complexas (BRZOZOWSKI *et al.*, 2005).

2.1.3 Mecanismos fisiológicos de defesa do sistema gástrico

Os mecanismos de defesa que atuam na porta de entrada dos xenobióticos no organismo são diversos, e entre eles encontram-se (Fig. 1):

- Os mastócitos e macrófagos residentes na lamina própria atuam como células sinalizadoras da presença de substâncias estranhas. Essas células são capazes de liberar uma grande quantidade de mediadores inflamatórios e citocinas que podem alterar o fluxo sanguineo da mucosa e aumentar o recrutamento de granulócitos para a região afetada (HOGABOAM *et al.*, 1993).
- Um epitélio especializado de forma a manter sempre as suas funções como barreira ao ácido gástrico e a outros agentes. A grande capacidade de proliferação do epitélio lhe confere habilidade de reparação ao dano epitelial e contribui para a resistência da mucosa gástrica às lesões (WALLACE, 2001).
- O muco também tem importante papel na prevenção da agressão mecânica ao epitélio, e fornece um microambiente sobre a área lesionada, que é rapidamente restituída (WALLACE, 2001), atuando principalmente como barreira física.
- O fluxo sanguineo contribui para a proteção gástrica por fornecer à mucosa: oxigênio, bicarbonato, substâncias nutritivas e por remover o dióxido de carbono, íons hidrogênio e difundir agentes tóxicos do lúmen gástrico (SORBYE; SVANES, 1994).
- As prostaglandinas apresentam efeito na motilidade, secreção e citoproteção do trato gastrintestinal. A secreção do muco e bicarbonato, a vasodilatação e a rápida regeneração epitelial são alguns dos componentes de defesa da mucosa que são regulados pelas prostaglandinas (WALLACE, 2008).
- O óxido nítrico apresenta papel chave na perfusão e regulação vascular por promover a vasodilatação pela sinalização da célula muscular lisa via cGMP. A produção constitutiva de NO é importante para manter a barreira protetora da mucosa gastrintestinal, e esse mecanismo protetor é devido à sua capacidade de aumentar o fluxo sanguíneo da mucosa e estabilizar a influência dos mastócitos (ALICAN *et al.*, 1996).

Figura 1- Mecanismo de defesa e injúria da mucosa gástrica.



Fonte: Adaptado de Robbins; Contran, 2005.

2.1.4 Epidemiologia das doenças gástricas

Nas últimas décadas a incidência da doença ulcerosa gástrica declinou no mundo ocidental. Entretanto, apresenta incidência que varia de 2 a 10/100.000 pessoas por ano, permanecendo como problema de saúde pública na sociedade moderna (KOMEN *et al.*, 2008). As complicações mais importantes relacionadas à doença ulcerosa gástrica são: hemorragia, observada clinicamente em 15-20% dos casos, e perfuração, em 7%, com incidência de 7 a 10/100.000 pessoas por ano – variável entre os países e mesmo entre regiões de um mesmo país (DIOGO-FILHO *et al.*, 2003). A mortalidade anual relacionada à doença ulcerosa é baixa, sendo ela consequente à complicações em pacientes com comorbidades ou do tratamento cirúrgico. Por outro lado, a morbidade dessa afecção tem sido reportada como entre 25% e 89%, com custos elevados. Entre os pacientes com úlcera duodenal, 6% a 11% apresentam perfuração e, entre aqueles com úlcera gástrica varia de 2% a 5% (KOCER *et al.*, 2007).

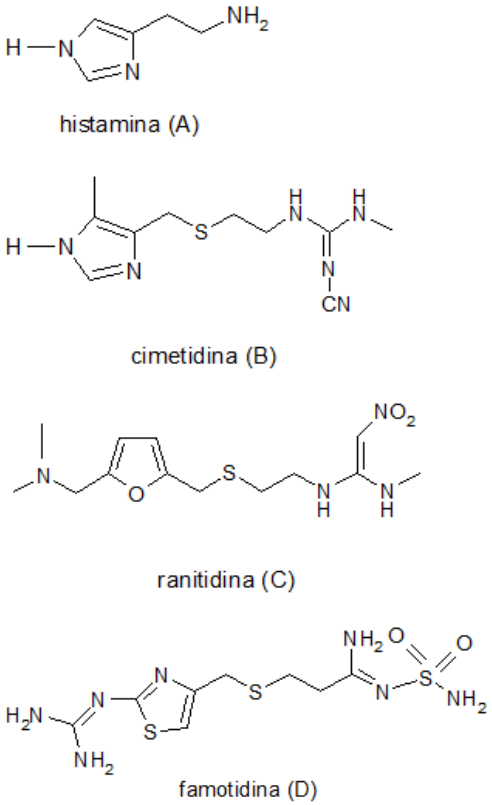
A faixa de idade predominante na qual a úlcera duodenal ocorre é entre 20 e 50 anos, enquanto que a gástrica é mais comum em paciente com mais de 50 anos (KOMEN *et al.*, 2008). A úlcera duodenal é mais frequente nas populações ocidentais, e as úlceras gástricas são mais comuns nos países orientais, particularmente o Japão (SIVRI, 2004).

2.1.5 Fármacos utilizados nos tratamentos dos distúrbios gástricos

Os fármacos utilizados no tratamento das úlceras ou distúrbios ácido-pépticos promovem a cicatrização da lesão e vários tipos podem ser utilizados (isoladamente ou em associações). De modo geral, as terapias estão diretamente ligadas à diminuição da secreção ácida.

- **Antagonistas de receptores H2** – cimetidina, ranitidina, famotidina (Fig. 2) - Os antagonistas H2 agem competindo com a histamina (Fig. 2) pela ligação com o receptor H2, o que também contribui para a diminuição da secreção ácida gástrica (BRUNTON *et al.*, 2007). Recentemente, essa classe de fármacos vem sendo substituída pelos inibidores da bomba de prótons, contudo, devido ao seu custo mais acessível ainda é utilizada (ANVISA, 2010).

Figura 2 – Estrutura química do aminoácido histamina (A) e a estrutura química de três moléculas de fármacos antagonista H2 [cimetidina (B), ranitidina (C), famotidina (D)].

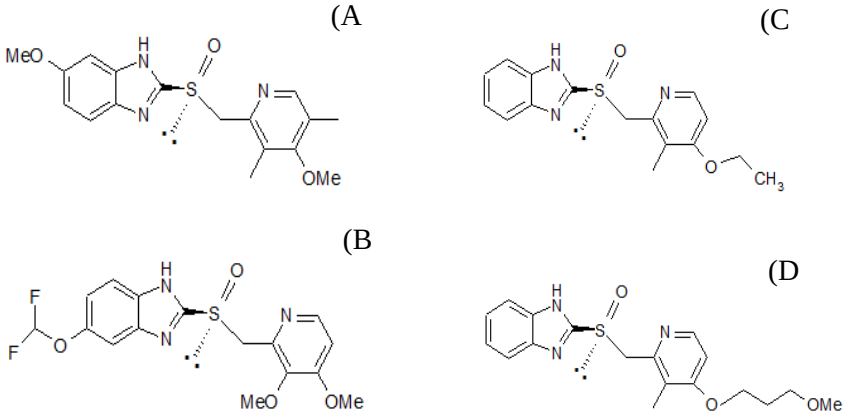


Fonte: adaptado de Brunton et al., 2007.

- **Inibidores da bomba de prótons (H^+ /K^+ APTase)** – Omeprazol, lansoprazol, rabeprazol, pantoprazol (Fig. 3). Atualmente estes são os fármacos de primeira escolha.

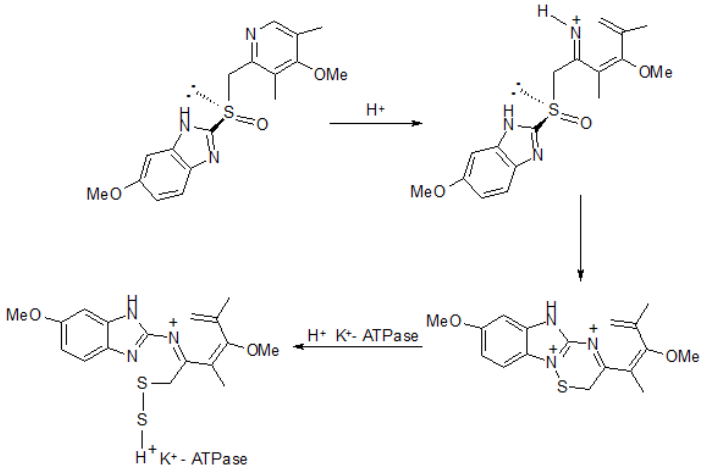
Após a sua absorção se difundem até as células parietais onde se acumulam nos canalículos secretores de ácido (BRUNTON *et al.,* 2007). Devido ao seu mecanismo de ação (Fig. 4) diminuem a secreção de HCl pela mucosa gástrica. Em casos de secreção ácida aumentada a mesma retorna ao nível normal com a administração desses inibidores irreversíveis da bomba de prótons (SCHUBERT, 2009), que podem ser associados ou não os antimicrobianos (a depender da presença de *H. pylori*).

Figura 3 – Estrutura química dos principais fármacos inibidores de bomba de prótons: omeprazol (A), pantoprazol (B), lansoprazol (C), rabeprazol (D).



Fonte: adaptado de Brunton *et al.*, 2007.

Figura 4 – Mecanismo de ativação do omeprazol. O omeprazol é uma base fraca, especificamente concentrada na secreção ácida dos canalículos das células parietais e quando ativada por um próton gera uma sulfenamida. Essa sulfenamida interage covalentemente com os grupos sulfidrilas do resíduo de cisteína no domínio extracelular da H + K + -ATPase – Cis813 – inibindo sua atividade.



Fonte: adaptado de Brunton *et al.*, 2007.

- **Antiácidos** – são fármacos utilizados de forma a aumentar as defesas da mucosa. São usados para aliviar a pirose e o desconforto abdominal. Neutralizam o ácido secretado, e são rapidamente absorvidos devido à sua alta solubilidade em água. Podem ser utilizados de forma isolada (hidróxido de alumínio, hidróxido de magnésio, magaldrato - hidróxido de alumínio e magnésio), em forma de misturas de antiácidos ou associados a outros fármacos (BRUNTON *et al.,* 2007).

- **Análogos de Prostaglandinas** – Misoprostol, análogo sintético da PGE1. Atua protegendo a mucosa gástrica através de efeitos que incluem: estimulação de secreção de muco e bicarbonato e aumento do fluxo sanguíneo no estômago (BRUNTON *et al.*, 2007). O misoprostol aumenta a produção de muco no estômago, contudo provoca também um aumento acentuado na contração de músculos lisos, principalmente no útero, induzindo o aborto.

- **Outros** – Sucralfato trata-se de um polímero que adere às células epiteliais e à área lesada formando uma barreira física de proteção, além de estimular a produção local de prostaglandinas e fator de crescimento epidérmico (BRUNTON *et al.*, 2007).

- **Tratamento da infecção por *Helicobacter Pylori***- os principais tratamentos indicados para a erradiação da bactéria e para a recuperação da mucosa gástrica podem ser divididos em: Terapia tripla – com a utilização de um inibidor da bomba de próton, um antibiótico e um antiprotozoário; Terapia quádrupla – com a utilização de um inibidor da bomba de prótons, um antiprotozoário, um antibiótico e um antiácido ou Terapia quádrupla com a utilização de um antagonista de receptor H2, um antiácido, um antiprotozoários, e um antibiótico (BRUNTON *et al.*, 2007).

2.2 MODELOS EXPERIMENTAIS DE LESÃO GÁSTRICA

Os estudos em animais de laboratório tentam recriar as formas de gastrite e úlcera gástrica que acometem os seres humanos. Contudo, muitas formas de gastropatia humana ainda não tiveram seus modelos desenvolvidos em animais. Os estudos animais, embora possuam a limitação tanto da etiologia quanto das diferenças estruturais e fisiológicas do estômago em relação aos seres humanos, têm a vantagem de permitir o estudo de gastrites e úlceras com origens especificas, como a gastrite

induzida por AINES e outros agentes nocivos, como substâncias necrotizantes incluindo ácidos, dano por substâncias quentes, estresse e etanol (SMITH, 1989).

2.2.1. Lesão gástrica induzida por etanol

O modelo de lesão gástrica induzida por etanol em roedores pode ser produzido de forma bastante confiável pela simples administração intragástrica de quantidades variáveis (0,5 a 2,0 mL) de etanol em diferentes concentrações (50 a 100%). Dependendo da quantidade de etanol, entre 10 e 40% da porção glandular do estômago de ratos e camundongos torna-se coberta de erosões hemorrágicas e úlceras após um período de 1 a 2 horas. Estudos de evolução temporal das lesões, neste modelo, entretanto, mostram que a maior parte do dano produzido pelo etanol ocorre entre 1 e 3 minutos após instilação no estômago (GLAVIN; SZABO, 1992).

O dano gástrico produzido pela administração aguda de etanol promove a formação de eritema e erosões superficiais, friabilidade e hemorragia microscópica da mucosa. Parte do etanol administrado é metabolizado pelas células da mucosa, resultando na formação de acetaldeídos tóxicos, mais relacionados à patogênese da gastrite alcoólica crônica do que da aguda (LIEBER, 1997).

O mecanismo de ação lesiva do etanol, portanto, é multifatorial (Fig. 5). As lesões são produzidas em consequência de variados fatores que interagem, e cada um destes fatores pode ser um alvo terapêutico em potencial. O etanol aumenta a produção de ânions superóxido, radicais hidroxila e a peroxidação lipídica da mucosa. Etanol induz também ao estresse oxidativo intracelular através da transição da permeabilidade e despolarização da mitocôndria, que precede a morte celular da mucosa. Sabe-se que o etanol promove significativa redução da concentração de grupamentos sulfidrílicos não proteicos dos tecidos animais, provavelmente pela formação de radicais livres. A mucosa responde à peroxidação lipídica produzida pelo etanol através da captação de radicais livres pelo GSH e outros grupamentos sulfidrílicos não proteicos. Em seres humanos, o processo inflamatório produzido por etanol está associado com a total depleção dos grupamentos sulfidrílicos (tanto proteicos quanto não proteicos) como a GSH e cisteína. Compostos exógenos contendo grupamentos sulfidrila protegem a mucosa gástrica de danos produzidos pelo etanol (PIHAN *et al.*, 1987; LOGUERCIO *et al.*, 1993; REPETTO; LLESUY, 2002).

O etanol também parece alterar a secreção ácida gástrica e interferir com a motilidade gástrica e intestinal. Em algumas espécies animais, a administração aguda de etanol, seja via oral, intragástrica, através de cânula de gavagem, ou mesmo via sanguínea promove alteração da secreção ácida. A resposta secretória ao etanol varia consideravelmente de acordo com a espécie e com as concentrações utilizadas. Por exemplo, bebidas com baixo teor de etanol, como cerveja e vinho, aumentam fortemente a secreção ácida gástrica e a liberação de gastrina na espécie humana. Em contraste, bebidas com alto teor alcoólico, como uísque e conhaque, não produzem tal estimulação ácida ou de gastrina em seres humanos. O mecanismo de ação deletéria do etanol parece ser tanto local (tópica), sobre a mucosa, quanto uma ação sistêmica, afetando a liberação de hormônios e a regulação das funções nervosas envolvidas na secreção ácida. Estudos em humanos e animais mostram que concentrações alcoólicas superiores a 10% causam rompimento da barreira mucosa e aumentam sua permeabilidade. Uma exposição prolongada ao etanol promove distúrbio da microcirculação levando a um progressivo desarranjo estrutural da mucosa (BODE & BODE, 1997).

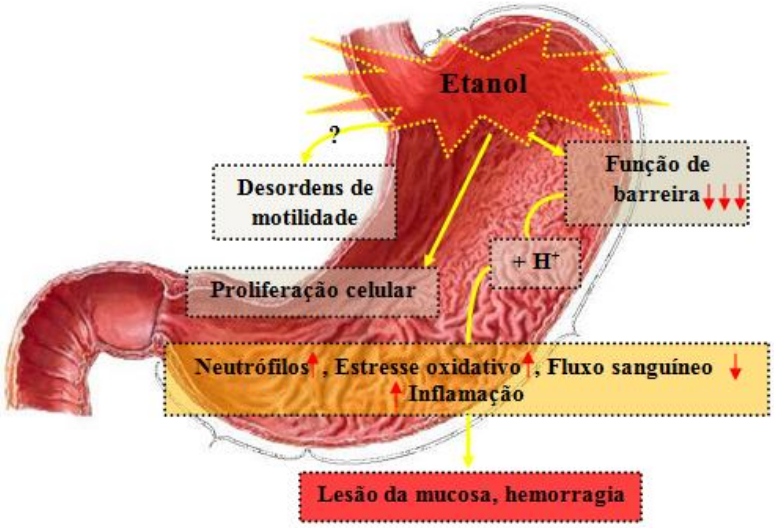
O etanol atua como agente pró-inflamatório agudo, estimulando a aderência leucocitária (neutrófilos) com consequente dano ao epitélio em baixas concentrações (10%), e lesões independentes de neutrófilos em altas concentrações (90 a 100%), mais relacionadas ao dano vascular precoce, com diminuição do fluxo sanguíneo da mucosa, isquemia e morte celular. O disparo do processo inflamatório que é produzido pelo etanol agudamente administrado é caracterizado pela liberação de mediadores, com ativação de granulócitos e produção consequente de proteases e formação de radicais livres (TEYSSEN; SINGER, 2003; SIEGMUND *et al.*, 2003).

O enfraquecimento da função de barreira da mucosa gástrica, gerando aumento da permeabilidade, promove modificação do potencial eletroquímico das membranas celulares, o que causa retro difusão dos íons H^+ através da mucosa lesionada (SIEGMUND *et al.,* 2003). A resposta da mucosa ao etanol também é refletida no aumento significativo da expressão de citocinas inflamatórias, entre elas o TNF-α (2,5 vezes), e no incremento da taxa de apoptose (9,4 vezes) das células epiteliais. Inibidores de bomba de prótons, como o omeprazol, não protegem a mucosa gástrica neste modelo experimental de lesão, porém, o uso de sucralfato, um polissacarídeo sulfatado que forma complexos com as proteínas mucosas adjacentes à úlcera (constituiem uma

barreira protetora contra a agressão do ácido gástrico e pepsina), promove redução significativa (95%) nas lesões pelo etanol (PIOTROWSKI *et al.*, 1997). Ranitidina, um antagonista de receptores de histamina tipo H_2, pode ou não promover proteção da mucosa gástrica em modelos de lesão por etanol, dependendo da dose do fármaco e da concentração do agente lesivo. Em doses acima de 50 mg/kg ocorre redução das lesões promovidas pelo etanol absoluto, abaixo disso, os estudos mostram certa ineficácia do fármaco (DEL-SOLDATO *et al.*, 1985; LEE *et al.*, 2002; SHEEBA; ASHA, 2006; CADIRCI *et al.*, 2007).

Os estudos sugerem que o etanol também promove redução da produção de PGs, desviando o equilíbrio do metabolismo do ácido araquidônico para a produção de leucotrienos, associados ao aumento da secreção ácida gástrica e desenvolvimento de lesões. Além disso, o etanol interfere com a atividade da musculatura do estômago e intestino, alterando o trânsito do alimento ao longo do TGI (TABUCHI & FURUHAMA, 1994; BODE & BODE, 1997).

Figura 5 – Multiplo mecanismo de ação do etanol absoluto na indução experimental de úlcera gástrica (aguda ou crônica).



Fonte: adaptado de Siegmund *et al.*, 2003.

2.2.2. Lesão gástrica induzida por Indometacina

O surgimento de lesões gástricas como resultado do tratamento com AINES é reconhecidamente um dos mais sérios efeitos adversos desta classe de medicamentos. A administração parenteral de AINES, incluindo o ácido acetilsalicílico e a indometacina representa um modelo animal simples e efetivo para o estudo da gastropatia induzida

por AINES. AINES reduzem o fluxo sanguíneo, promovendo redução do pH levando em última instância á formação de lesões hemorrágicas, incluindo úlceras. Os estudos indicam que os AINES induzem a formação de lesões gástricas por pelo menos dois mecanismos. O primeiro é o bem caracterizado bloqueio das enzimas COX, inibindo a produção endógena de PGs. O segundo mecanismo é chamado captura de íons e resulta da dissociação ácida dos AINES (pKa – 3,5 a 4,0) no ambiente intracelular neutro (pH – 7,00) das células mucosas. Os AINES, no estado ionizado, são solúveis em água e ficam retidos dentro das células, criando um gradiente de concentração que favorece o movimento dos íons dissociados dos ácidos orgânicos para dentro da mucosa gástrica. As alterações de permeabilidade resultam do influxo de H^+ e efluxo de íons Na^+ e K^+ para o lúmen gástrico (GLAVIN & SZABO, 1992; FIORUCCI *et al.,* 2001).

Atualmente, sabe-se que a inibição tanto da COX-1 quanto da COX-2 é necessária para o desenvolvimento das erosões gástricas após administração de AINES em ratos. Nem inibidores seletivos COX-1 ou COX-2 isoladamente causam dano macro ou microscopicamente detectável quando administrados em doses que inibem seletivamente estas enzimas *in vivo*. Mecanismos pelos quais a inibição da COX-1 e COX-2 contribuem para a formação da erosão são objeto de estudos atualmente. A aderência leucocitária induzida pelos AINES ao endotélio vascular contribui para geração do dano da mucosa. Esta adesão se dá, em parte, pela supressão da produção tônica de prostaglandinas (como a PGI_2) pelo endotélio vascular. Embora os numerosos registros experimentais de que a COX-1 é constitutivamente expressa, a habilidade dos AINES em estimular a aderência de neutrófilos era considerada como sendo causada pela supressão da COX-1 no endotélio vascular. Entretanto, inibidores seletivos COX-2 como celecoxib disparam significativa adesão leucocitária em vênulas mesentéricas, enquanto os inibidores seletivos COX-1, não promovem tal adesão. O decréscimo do fluxo sanguíneo gástrico após administração de AINES foi documentado em animais e humanos, e tem sido sugerido como fator significativo para a patogênese da injúria gástrica assim como para o modelo que se utiliza de etanol. Os AINES possuem outras ações não correlacionadas à supressão da COX que contribuem para o aparecimento das lesões. Por exemplo, muitos AINES exercem efeitos irritantes tópicos que podem contribuir para a injúria gástrica (WALLACE *et al.,* 2000).

Indometacina é um derivado indólico com atividade anti-inflamatória, analgésica e antipirética utilizado em diversas condições inflamatórias crônicas, em virtude da sua

efetiva supressão da dor, febre e edema (SÜLEYMAN *et al.,* 2010). O dano gástrico induzido por indometacina (inibidor inespecífico COX) e outros AINES também está relacionado à produção de EROs e peroxidação lipídica. O acúmulo de hidroperóxidos lipídicos cresce paralelamente ao desenvolvimento das lesões (YOSHIKAWA *et al.*, 1993). A fonte de radicais de oxigênio não foi ainda determinada, mas possivelmente, os EROs são derivados de neutrófilos no início da patogênese das lesões, pois o dano induzido por indometacina pode ser significativamente reduzido pela depleção de neutrófilos da circulação. Os radicais superóxido podem interagir com peróxido de hidrogênio, na presença de ferro, para gerar radicais hidroxila, que é um dos mais tóxicos reagentes implicados na peroxidação lipídica, pela abstração de átomos de H^+ de radicais metileno dos ácidos graxos poli-insaturados da membrana plasmática (YOSHIKAWA *et al.,* 1993).

Os AINES também reduzem a secreção de muco e de bicarbonato, que é regulada pela síntese de PGs, tendo em vista que ambos os fatores são relacionados com a prevenção de dano físico e químico, a sua administração. A utilização dessa classe farmacológica aumenta a susceptibilidade da mucosa gástrica às agressões por ela exposta (FIORUCCI *et al.,* 2001). Existem ainda indícios de que os AINES podem agir através do bloqueio de receptores α2-adrenérgicos o que seria mais uma circunstância responsável pelo aumento de fatores agressivos à mucosa gástrica (SÜLEYMAN *et al.*, 2010).

2.3 PLANTAS MEDICINAIS

A biodiversidade dos vegetais constitui uma grande riqueza em potencial para a saúde humana, sendo as plantas fontes de produtos naturais biologicamente ativos. O uso destas em práticas populares e tradicionais como medicamentos caseiros e comunitários, processo conhecido como medicina popular, nos remete a seus princípios ativos como importantes substratos para o desenvolvimento de medicamentos (MACIEL *et al.*, 2002; CALIXTO, 1997).

A evolução do conhecimento científico sobre as plantas e sua utilização pelo homem têm ocorrido através dos tempos. Civilizações primitivas perceberam a existência de plantas comestíveis e dotadas de toxicidade que, ao serem utilizadas no

combate às doenças, revelavam empiricamente seu potencial curativo (BARROS, 2008).

Antes do século XIX, a maioria dos recursos terapêuticos existentes era constituída por plantas e extratos, sendo estes a parte qualitativamente mais importante da terapêutica na época. Com o aumento progressivo do setor industrial após a Segunda Guerra Mundial, os "remédios vegetais" foram gradativamente substituídos nas farmácias por medicamentos com substâncias ativas extraídas ou derivadas sinteticamente de plantas (SIMÕES *et al.,* 2004).

O contexto etnobotânico e etnofarmacológico vêm resgatando os conhecimentos tradicionais passados de geração em geração e contribuindo para descobertas de novas drogas com princípios ativos para o tratamento de enfermidades. A maioria dos povos ou etnias globais usam plantas medicinais ou seus derivados, no tratamento dos males que afetam a saúde. Nos países em desenvolvimento, é uma prática comum com um contingente de 80 % da população dependente da medicina popular (MELO, 2007).

Os estudos etnobotânico e etnofarmacológico, voltados ao Brasil são de extrema importância, uma vez que o país apresenta a maior diversidade genética vegetal do mundo, contando com mais de 55.000 espécies catalogadas (SIMÕES *et al.,* 2004), onde 99,6% são desconhecidas quimicamente (GOTTLIEB *et al.,* 1998).

No Brasil, tem crescido o interesse pelo estudo das plantas medicinais em resposta a tendência mundial de preocupação com a biodiversidade, pautada na ideia de desenvolvimento sustentável (MOSCA, 2009). É comum, no comércio brasileiro a venda de uma quantidade expressiva de espécies vegetais que se destinam ao tratamento de diversas enfermidades, e, recentemente aparecem como componentes de muitos produtos industrializados, comercializados como drogas vegetais e/ou fitoterápicos (MELO, 2007). O uso de plantas medicinais e da fitoterapia, encontram-se em ascensão mundial e endossam um mercado promissor (NASCIMENTO *et al.,* 2005) com cerca de 50% de plantas utilizadas na alimentação, 25% na indústria cosmética, 20% na indústria farmacêutica e 5% em outras atividades, estimando-se em 10.000 o número de espécies vegetais medicinais (MELO, 2007).

Segundo Calixto e Yunes (2001), cerca de 50% dos medicamentos utilizados são de origem sintética e 25% de origem vegetal, isolados diretamente ou sintetizados a partir de um precursor vegetal. A apesar desse contingente, 20% das plantas encontradas no mundo ainda estão sendo submetidas a algum teste biológico e/ou farmacológico

(SUFFREDINI *et al.*, 2004), fato que vem sendo estimulado pela crescente procura da população mundial (60 – 80%) por fitoterapia no tratamento de várias doenças (SCOPEL, 2005).

Contudo, apesar de toda importância atribuída às plantas, o seu potencial terapêutico e toxicológico, no que concernem mecanismos de ação, ainda são pouco explorados, pois apenas recentemente estas se tornaram objeto de estudo científico (BARROS, 2008).

2.3.1 Plantas medicinais com atividade gastroprotetora

O estudo de plantas medicinais que possuam ação sobre o trato gastrintestinal assume grande importância uma vez que os medicamentos atualmente disponíveis para o tratamento destes distúrbios apresentam efeitos colaterais. O uso de antiácidos pode causar constipação e diarreia (RANG *et al.*, 2001) e o uso crônico de fármacos antisecretores tais como os antagonistas dos receptores H2 e os inibidores da bomba de prótons podem gerar problemas como gastrinemia (GARNETT, 1998). Além disso, um estudo realizado por Arrais *et al.*, (1997) demonstrou que a procura por medicamentos que tenham ação sobre o aparelho digestivo e o metabolismo é de 24%, indicando que grande parte da população sofre de distúrbios gástricos, existindo grande necessidade de fármacos que apresentem menos efeitos colaterais e maior eficácia. A busca por princípios ativos que possam ser isolados ou servir de modelo para a síntese de novos fármacos é crescente e as pesquisas nesta área são promissoras.

No continente americano 58 espécies apresentam atividade gastroprotetora, distribuídas em 37 famílias, entre elas Turneraceae (2 espécies), Fabaceae (6 espécies), Celastraceae (3 espécies) e Asteraceae (13 espécies). A maioria é encontrada no Brasil, mas a flora de vários países (Argentina, Cuba, Estados Unidos, Santa Lúcia, Costa Rica, Bolívia, Equador e México) também apresenta espécies com tal atividade avaliada pelo menos em ensaios pré-clínicos (FALCÃO *et al.*, 2008).

Outros estudos utilizando plantas medicinais mostram atividade gastroprotetora – sejam estudos etnofarmacológicos ou pré-clínicos em roedores – em espécies tão diferentes como *Piper nigrum* Linn. (Piperaceae), *Dodonaea viscose* (Sapindaceae), *Mouriri pusa* (Melatomataleae), *Ficus arnottiana* (Moraceae), *Eruca sativa* L. (Brassicacae), *Cissampelos mucronata* (Menispermaceae), *Allium sativum* Linn. (Liliaceae), *Keilmeyera coriacea* (Gultiferae), *Terminalia chebula* (Combretaceae),

Garcinia cambogia (Gaertnaceae) (RANI *et al.,* 2010), e *Avicennia alba* (Acanthaceae) (AL-ATTAR, 2011).

2.3.2 As plantas medicinais e a legislação no ambito regulatório

Os medicamentos derivados de plantas medicinais, comumente denominados Fitoterápicos, são tão eficazes quanto os medicamentos produzidos com ativos oriundos de síntese química, entretanto, a transformação de uma planta num medicamento deve priorizar a preservação da integridade química dos princípios ativos e, consequentemente, a ação farmacológica do vegetal, garantindo a permanência da ação farmacológica. A produção de Fitoterápicos requer, portanto, estudos prévios relativos a aspectos botânicos, agronômicos, fitoquímicos, farmacológicos e toxicológicos, associados ao desenvolvimento de metodologias analíticas (TOLEDO *et al.,* 2003).

A regulamentação dos medicamentos fitoterápicos industrializados no Brasil é realizada pela ANVISA, órgão federal que registra medicamentos e demais produtos destinados à saúde, intervindo nas atividades de produção, distribuição, comercialização, publicidade, consumo e descarte de medicamentos, além de análises laboratoriais. O intuito deste é o gerenciamento dos possíveis riscos à saúde em todas as fases da cadeia dos produtos, onde se incluem os fitoterápicos. É a ANVISA a responsável pelo registro de todos os fitoterápicos, antes de sua comercialização, para garantir que a população tenha acesso a medicamentos seguros, com eficácia e qualidade comprovada. Cada registro tem validade de cinco anos, devendo ser renovado por períodos sucessivos, conforme determinado na Lei nº 6.360/76, que dispõe sobre os produtos submetidos ao controle da Vigilância Sanitária (CARVALHO *et al.,* 2007).

A RDC nº 48/04 é a norma regulatória para o registro de fitoterápicos. Estabelece todos os requisitos necessários para a concessão do registro, baseados na garantia de qualidade. As avaliações abrangem a matéria-prima vegetal, os derivados de droga vegetal e o produto final, o medicamento fitoterápico. É exigido ainda Certificado de Boas Práticas de Fabricação e Controle para as linhas de produção da empresa. Cabe ressaltar que as normas exigidas para a produção de fitoterápicos são as mesmas estabelecidas para os demais medicamentos. A citada norma ainda prevê as formas de se comprovar segurança e eficácia dos fitoterápicos, incluindo os estudos etnofarmacológicos (CARVALHO *et al.,* 2007).

Outros regulamentos dispõem sobre produção, registro e comercialização de medicamentos, inclusive fitoterápicos, tais como: informações de bula (Portaria nº 110/97 e RDC nº 140/03), modelos e dizeres de embalagens (RDC nº 333/03); restrição de venda (RDC nº 138/03); publicidade (RDC nº 102/00); testes de comprovação de qualidade, incluindo Guia para Realização de Estudos de Estabilidade (RE nº 01/05) e Guia para Realização de Validação de Metodologia Analítica (RDC nº 899/03) (CARVALHO *et al.*, 2007).

2.4 FAMÍLIA ASTERACEAE

2.4.1 Aspectos botânicos

As Asteraceae (Dumortier) possuem distribuição cosmopolita e constituem a maior família de Eudicotiledôneas, com 1.620 gêneros e 23.600 espécies (STEVENS, 2012). No Brasil, a família Asteraceae apresenta 274 gêneros, 2053 espécies, 22 subespécies e 39 variedades (NAKAJIMA *et al.*, 2014).

As espécies pertencentes à família Asteracea podem apresentar porte herbácea, subarbustos, arbustos, pequenas árvores ou lianas, com folhas alternas ou opostas, raramente verticiladas, simples, margem inteira ou serreada. As inflorescências são capítulos envolvidos por brácteas, formando um invólucro. As flores são todas iguais entre si, ou diferenciadas em flores do raio e flores do disco. As primeiras são altamente modificadas, podendo ser estéreis e possuir corola hipertrofiada, enquanto as flores do disco são bissexuadas ou raramente unissexuadas. O fruto é do tipo cipsela, com papilho geralmente persistente (SOUZA; LORENZI, 2008).

2.4.2 Importância econômica

Do ponto de vista econômico, cerca de 40 espécies têm importância direta na alimentação humana, como *Lactuca sativa* (alface) e *Cichorium intybus* (chicória), e indireta na obtenção de produtos, como *Helianthus annuus* (girassol), *Matricaria recutita* (camomila) e *Baccharis trimera* (carqueja). Espécies silvestres têm potencial nutricional, e muitas outras são de interesse tecnológico ou ornamental, e centenas produzem metabólitos secundários de uso farmacêutico ou industrial ou ainda fornecem néctar e pólen para a apicultura e também forragem para a produção pecuária (VITTO; PETENATTI, 2009).

Muitas plantas dessa família são conhecidas pelas suas propriedades medicinais e diversas espécies possuem atividade analgésica, antiinflamatória e antimicrobiana comprovadas (LORENZI; MATOS, 2002). Por produzirem compostos químicos bastante promissores, são de grande interesse para a indústria farmacêutica (ARAÚJO *et al.,* 2008).

2.4.3 Aspecto fitoquímico

Quimicamente a família Asteraceae é conhecida por produzir principalmente poliacetilenos, sesquiterpenoides, diterpenoides, triterpenoides, flavonóides, cumarinas, benzofuranos e benzopiranos (EMERENCIANO, 1998).

Inúmeros estudos com espécies dessa família apresentaram o isolamento de uma variedade de metabólitos secundários, dentre eles: diterpenos, triterpenos, cumarinas, sesquiterpenos, fenilpropanóides poliacetilenos, lactonas sesquiterpênicas, alcalóides, óleos essenciais, antocianinas (VERDI *et al.,* 2004), com destaque aos flavonóides, alocados como importantes marcadores quimiotaxonômicos, além de sua reconhecida importância para a medicina, no tratamento e prevenção de várias doenças (HARBORNE; WILLIAMS, 2000)

Várias espécies pertencentes a família Asteraceae apresentam atividade antiulcerogênica em diferentes modelos experimentais. Alguns exemplos podem ser citados: *Achillea millefolium* (BAGGIO *et al.,* 2003), *Mikania laevigata* (BIGHETTI, 2005), *Senecio brasiliensis* (TOMA *et al.,* 2004), *Franseria artemisioides*, *Bacharis genistelloides*, *Bacharis rubricaulis*, *Bacharis illinita* (GONZALES, 2000; VERDI, 2004). O extrato obtido a partir das folhas e frutos de *Sapindus saponaria* L., rico em triterpenos pentacíclicos, apresentou atividade antiulcerogênica e anti-secretória gástrica, diminuindo a concentração de ácido clorídrico e o pH gástrico (MEYER *et al.*, 2002). Lactonas sesquiterpênicas com atividade antiulcerogênica foram isoladas de *Artemisia douglasiana* (GUARDIÃ; GUZMAN, 1994). Dihidro-epideoxiartenuína b, isolada de *Artemisia annua*, apresentaram ação antiulcerogênica, estimulando a produção de muco através do aumento dos níveis gástricos de prostaglandinas (FOGLIO *et al.,* 2002; DIAS, 2004). *Ecabet sodium* demonstrou capacidade de reepitelização retal, pelo aumento de prostaglandina E2 em paciente submetido à radioterapia (AKIKO *et al.,* 2009).

Portanto, as plantas medicinais e seus metabólitos secundários, como terpenos e flavonóides, apresentam importância como agentes terapêuticos, no tratamento de desordens gastrintestinais tais como úlceras, gastrites e diarréias (SCHMEDA-HIRSCHMANN; YESILADA, 2005).

2.4.3.1 Terpenos

Os terpenos representam uma classe de metabólito secundário amplamente distribuído no reino vegetal. Possuem uma composição molecular típica $(C5H8)_n$, sendo formados por duas ou mais unidades isoprênicas. Estudos indicam que di, tri e tetraterpenos são biossintetizados na célula dentro dos cloroplastos (NABETA *et al.*, 1995).

Estes compostos representam a segunda classe com maior número de constituintes ativos obtidos de plantas, perdendo apenas para os flavonóides. Eles são divididos em monoterpenos (10 carbonos), sesquiterpenos (15 carbonos), diterpenos (20 carbonos), sesterpenos (25 carbonos), triterpenos (30 carbonos) e tetraterpenos (40 carbonos) (SEIGLER, 1981; DI-STASI, 1996).

Há varias décadas as propriedades farmacológicas dos terpenos vêm sendo determinadas, entre elas: antibiótica (NAKANISHI, 1974), antitumoral (SENILH *et al.*, 1998), anti-reumática (GU *et al.,* 1995) e antiulcerogênica (SHIRAKABE *et al.,* 1995). Especificamente as lactonas sesquiterpênicas, diterpenos e triterpenos foram avaliados cientificamente quanto ao seu potencial antiulcerogênico (Quadro 1).

Quadro 1 – Sumário de alguns dos principais terpenos descritos na literatura com atividade antiulcerogênica

COMPOSTO	CLASSIFICAÇÃO	ORIGEM	Fonte
***Trans*-crotonina**	Lactona sesquiterpênica	*Croton cajucara*	Hiruma-Lima *et al.*, 2002
Onodorpicrina	Lactona sesquiterpênica	*Arctium lappa*	Almeida, 2005
Ferruginol	Diterpeno	*Prumnopitys andina*	Rodriguez *et al.*, 2006
Ácido Oleanólico	Triterpeno	*Fabiana imbricata*	Astudillo *et al.*, 2002
Ácido Glicirretínico	Triterpeno	*Glycyrrhiza glaba*	Doll & Hill, 1962
Theasaponina	Saponina triterpenica	*Camellia sinensis*	Morikawa *et al.*, 2006

2.4.3.2 lactonas sesquiterpênicas (LS)

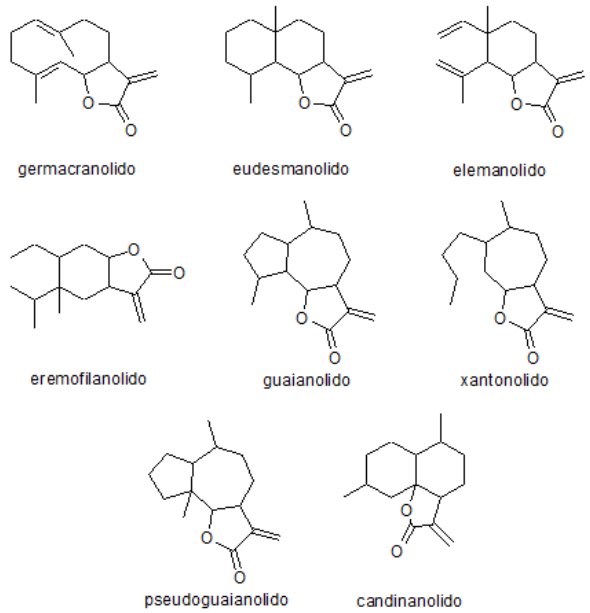
Lactonas sesquiterpênicas (LS) são compostos de grande ocorrência na natureza e representam um importante grupo de metabólitos secundários da família Asteraceae. As LS estão amplamente distribuídas nas plantas e mais de 7000 estruturas químicas

dessa classe foram descritas (MACIAS *et al.,* 2006). Devido ao seu amplo espectro de atividade biológica, as LS constituem uma classe de substâncias com potencial para utilização na medicina, destacando-se as atividades citotóxicas e antitumoral, antibacteriana, antiinflamatória, esquistossomicida, antimalárica e antifúngica. Contudo, algumas LS são consideradas altamente tóxicas (repina, helenalina) (HEINRICH, 1998; ZHANG *et al.,* 2005).

Os efeitos gastroprotetores das LS estão bem documentados e os mecanismos de proteção apontados pelos estudos envolvem efeito antioxidante em virtude de estruturas nucleofílicas presentes nas moléculas das LS, estimulação da secreção de muco gástrico e diminuição da secreção de ácido clorídrico (GIORDANO *et al.,* 1990; GUARDIA *et al.,* 1994).

Quanto a estrutura química as LS podem ser classificadas pelos seus carboesqueletos. Há quase 100 tipos de carboesqueletos, porém a maioria das LS, cerca de (87%), se enquadram em sete tipos de carboesqueletos conhecidos como germacranolídeos, elemanolídeos, eudesmanolídeos, eremofilanolídeos, guaianolídeos, xantanolídeos, pseudoguaianolídeos e cadinanolido (Fig. 6). Biogeneticamente, o carboesqueleto de germacrano é o precursor dos demais (SCHMIDT, 2006).

Figura 6 – Esqueleto básico das principais lactonas sesquiterpênicas



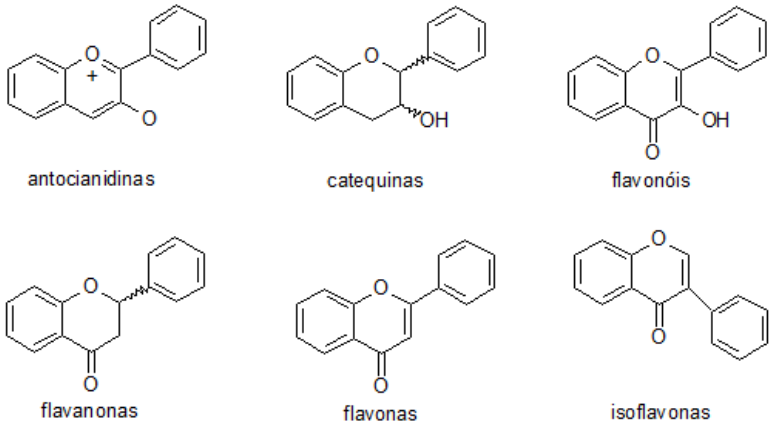
Fonte: adaptado de Schmidt, 2006

Os guaianolídeos representam um dos mais amplos grupos de LS com cerca de quinhentos compostos naturais conhecidos. Devido ao amplo espectro de suas atividades biológicas e sua baixa disponibilidade em fontes naturais, técnicas sintéticas para o preparo de guaianolídeos têm sido muito investigadas nos últimos anos (BARGUES *et al.*, 2002).

2.4.3.3 Flavonóides

Os flavonóides são metabólitos secundários de plantas biossintetizados a partir da via dos fenilpropanóides. Representam um dos grupos mais importantes e diversificados entre os produtos de origem vegetal e são amplamente distribuídas no reino vegetal. Sua distribuição depende de diversos fatores de acordo com a fila/ordem/família do vegetal, bem como da variação das espécies. Geralmente, flavonóides encontrados nas folhas podem ser diferentes daqueles presentes nas flores, nos galhos, raízes e frutos. O mesmo composto ainda pode apresentar diferentes concentrações dependendo do órgão vegetal em que se encontra (SIMÕES *et al.*, 2004). Quimicamente os flavonóides possuem estrutura marcada pela presença de um esqueleto com 15 átomos de carbono na forma C_6-C_3-C_6, e são divididos em classes dependendo do estado de oxidação do anel central de pirano (IKAN, 1991). São identificados como substâncias compostas por um núcleo comum de fenilcromanona com substituição em uma ou mais hidroxilas, incluindo derivados ligados a açucares (BIRT, HENDRICH, WANG, 2001). A figura 7 apresenta a estrutura química dos principais tipos de flavonóides (MOLNÁR-PERL; FÜZFAI, 2005).

Figura 7 – Esqueleto básico dos principais flavonoides



Fonte: adaptado de Molnár-Perl e Füzfai, 2005

2.4.3.3.1 - Flavonóides e atividades biológicas

Ensaios biológicos usando combinações isoladas revelam que os flavonóides exibem uma grande ação sobre os sistemas biológicos demonstrando atividade antimicrobiana, antiviral, antiulcerogênico, antioxidante, citotóxico, antineoplásico, antihepatotóxico, antihipertensivo, hipolipidêmico, anti-inflamatório, antiplaquetário (BENAVENTE-GARCÍA *et al.*, 1997, VAN-DAM; NAIDOO; LANDBERG, 2013; SIMÕES *et al.*, 2004; HAVSTEEN, 2002; MIDDLETON, KANDASWAMI, THEOHARIDES, 2000; VEITCH; GRAYER, 2008)

Harborne e Williams (2000) demonstraram que o grupo carbonila em C-4 e a dupla ligação entre C-2 e C-3 desempenham um importante papel na ação antioxidante dos flavonóides. Além disso, a configuração das hidroxilas no anel é determinante no processo de eliminação dos radicais livres.

As diferenças na atividade antioxidante de flavonóides por polihidroxilação ou polimetoxilação ocorrem, provavelmente, devido a diferenças nas configurações estruturais dos radicais livres. Após a doação de grupos hidroxila e metila pelos flavonóides esses radicais livres perdem sua reatividade, dessa forma não são capazes de atacar biomoléculas do organismo (HEIM; TAGLIAFERRO; BOBILYA, 2002).

Certos flavonóides podem bloquear os processos biosintéticos dos eicosanoides, inibir processos mitóticos, interações célula-célula, incluindo possíveis efeitos na adesão molecular. O mecanismo de inibição exercido pelos flavonóides sobre as enzimas lipoxigenase está sendo extensivamente pesquisado (NIJVELDT *et al.*, 2001). Flavonóides como a quercetina e a apigenina têm demonstrado possuir ação antiinflamatória por causar inibição de COX-2 (MUTOH *et al.*, 2000; RASO *et al.*, 2001). Segundo Friesenecker, Tsai, Intagliatta, (1995), flavonóides, como a quercetina e a luteolina, podem reduzir a ativação do sistema complemento, diminuindo a adesão de células inflamatórias ao endotélio, resultando em uma redução da resposta inflamatória.

Flavonóides e isoflavonóides podem inibir o ciclo celular e induzir a apoptose, linhagens de células. O flavonóide quercetina bloqueia o ciclo celular em G1/S de células cancerosas de cólon. Ele também induz apoptose, resultado da fragmentação nuclear e condensação da cromatina nuclear (REDDY, ODHAV, BHOOLA, 2003). Marchand (2002) descreveu que em modelos *in vitro*, flavonóides tem mostrado afetar sinalização celular e a progressão do ciclo celular. Genisteína e quercetina inibem a proteína tirosina quinase que também está envolvida na proliferação celular. Apigenina,

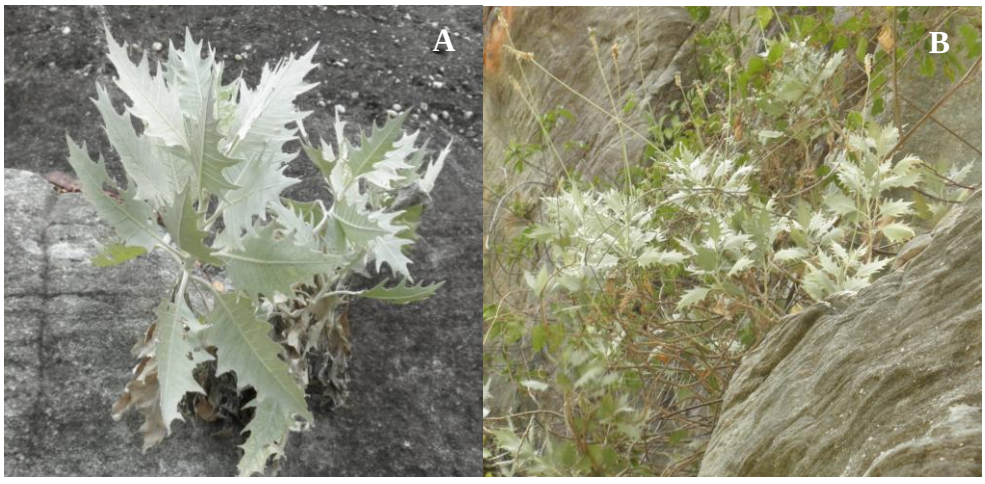
luteolina e quercetina mostraram-se eficazes no processo de morte celular, impedindo a progressão do ciclo celular através do mecanismo dependente de p-53 (REDDY; ODHAV; BHOOLA, 2003).

2.4.4 *Chresta martii* (DC.) H. Rob.

Atualmente, considera-se que o gênero *Chresta* é composto por 12 espécies (*Chresta amplexifolia*, *Chresta harleyi*, *Chresta martii*, *Chresta pinnatifida*, *Chresta curumbensis*, *Chresta speciosa*, *Chresta scapigera*, *Chresta angustifolia*, *Chresta plantaginifolia*, *Chresta sphaerocephala*, *Chresta exsucca* e *Chresta pycnocephala*) que ocorrem no cerrado, caatinga e campos rupestres no planalto brasileiro. É caracterizado genericamente como composto por ervas perenes ou arbustos, contendo de 2 a 12 floretes por cabeça e capítulos densamente congestos, arranjados em glomérulos solitários ou corimbosos (ROBINSON, 1999; ROQUE *et al.*, 2008).

A espécie *Chresta martii* (Fig. 8A e 8B) foi objeto de poucos estudos e existe ainda uma polêmica sobre sua real denominação e posição no cladograma das Asteraceae, em virtude de aspectos filogenéticos considerados de forma diferente por diferentes correntes da botânica, de modo que alguns autores a consideram um ramo extra e outros, sinonímia de *Argyrovernonia harleyi*. Entretanto, esta última denominação não consta na Lista de Espécies da Flora do Brasil (LISTA DE ESPÉCIES DA FLORA DO BRASIL, 2012).

Figura 8 – Espécime de *Chresta martii* fotografada na região de Xingó-Sergipe-BR durante expedição de coleta em Janeiro de 2011. Observa-se coloração cinza-prateada típica das suas folhas (A), bem como o substrato rochoso onde a espécie fixa-se e desenvolve-se (B).



Fonte: Franco, 2014

A planta é caracterizada por conter glomérulos cônicos a campanulados, folhas pecioladas de formato ovalado-romboide, com 30 a 40 cabeças (Fig. 9A), 12 a 13 floretes e uma corola medindo de 12 a 15 mm (Fig. 9B), com plumagem (*indumentum*) prateada-acinzentada (Fig. 8A) (ROBINSON, 1999; ROQUE *et al.*, 2008). A espécie apresenta apenas dois estudados experimentais abordando sua segurança e eficácia (SILVA *et al.*, 2012 e 2013), embora alguns estudos etnofarmacológicos tenham demonstrado sua utilização popular no tratamento de doenças do trato gastrointestinal, fígado e até malária (ALMEIDA *et al.*, 2005; ALMEIDA *et al.*, 2006; AGRA *et al.*, 2008).

Figura 9 – Aspecto da inflorecência da espécime de *Chresta martii*

Fonte: Franco, 2014

Dessa forma, o presente estudo objetivou avaliar a toxicidade aguda (*in vivo* e *in vitro*), atividade gastroprotetora dos extratos e frações semipurificas de *Chresta martii*, identificar a classe química e elucidar estruturalmente seu composto majoritário, proporcionando assim mais respaldo para a utilização dessa espécie através do conhecimento científico no que concerne: segurança, qualidade e eficácia.

3 OBJETIVOS

3.1 Geral:

Avaliar a toxicidade aguda (*in vivo* e *in vitro*) e atividade gastroprotetora dos extratos e frações semipurificas das partes aéreas de *Chresta martii* e identificar seu (s) composto (s) majoritário (s).

3.2 Específicos:

a) Obter extratos orgânicos (ciclohexânico, acetato de etila e etanólico) e frações semipurificadas de *Chresta martii*;
b) Avaliar a toxicidade aguda (*in vivo* e *in vitro)* e genotoxidade (*in vivo*) dos extratos orgânicos e frações semipurificadas de *C. martii;*
c) Rastrear a atividade gastroprotetora entre os extratos e frações semipurificadas de *C. martii*;
d) Obter compostos puros oriundos da fração semipurificadas que apresentar melhor atividade gastroprotetora;
e) Identificar o (s) composto (s) bioativo (s) majoritário (s) de *C. martii*;

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4 RESULTADOS

4.1 Artigos Derivados da Tese

A seguir serão apresentados dois artigos, sendo o primeiro intitulado "*Evaluation of the acute toxicity, cytotoxicity and genotoxicity of Chresta martii (Asteraceae)*" que foi aceito para publicação pelo **Journal of Toxicology and Environmental Health, Part A: Current Issues** e o segundo intutulado "*Study of the gastroprotective effect of extracts and semi-purified fractions of Chresta martii DC. and identification of its principal compounds*" submetido a publicação pelo **Evidence-Based Complementary and Alternative Medicine.**

4.1.1 ARTIGO ACEITO PELO JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH, PART A: CURRENT ISSUES.

Fator de impacto: 1.834; Qualis CAPES área de Biotecnologia: B2

EVALUATION OF THE ACUTE TOXICITY, CYTOTOXICITY AND GENOTOXICITY OF *Chresta martii* (ASTERACEAE)

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Running title: Toxicity evaluation of *Chretia martii*

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ABSTRACT

Chresta martii (Asteraceae), found in the Xingó region, northeastern Brazil, is used in the treatment of gastrointestinal (GIT) and liver disorders and malaria. However, there are few studies regarding efficacy and safety of use for this species. Thus, the objective of this study was to determine in *vivo* acute toxicity and *in vitro* cytotoxicity of organic extracts of *C. martii* as well as *in vivo* genotoxicity of its semi-purified fraction. Dried aerial parts of *C. martii* were extracted using three organic solvents (cyclohexane -ECCm, ethyl acetate -EACm and ethanol - EECm) and these extracts were examined for acute toxicity (50-2000 mg/kg; i.p. or p.o.) and cytotoxicity (50 μg/ml) in carcinogenic human cell lines (HL-60, NCIH-292 and MCF-7). The EACm, which showed evidence of toxicity (*in vivo* and *in vitro*), was fractionated on a silica column, yielding 4 fractions (F1 – F4). The F1 was utilized for genotoxicity (50 mg/kg; i.p.), by *in vivo* micronucleus (MN) assay. ECCm showed no

indication of acute toxicity or occurrence of death, while the LD50 estimated for the extracts (EACm and EECm) was 500 mg/kg; p.o. and 200 mg/kg; i.p. The EACm (50 μg/ml) inhibited growth of tumor cells HL-60 (96.54%), NCIH-292 (73.43%) and MCF-7 (15%). The F1 fraction induced MN formation in polychromatic erythrocytes of Swiss Webster mice. Organic extracts from *C. martii* exhibited acute toxicity classified as mild to moderate, in addition to cytotoxicity (*in vitro*) while F1 semi-purified fraction induced genotoxicity (*in vivo*).

Keywords: *Chresta martii*, gastroprotective, toxicity, safety of use.

INTRODUCTION

Although the importance of plant species for therapeutic applications is well-established, studies on certain plant-induced toxicity are scarce. In this regard *Chresta martii* (DC.) H. Rob. (Asteraceae), a species found in the Xingó (semi-arid) region of Sergipe, northeastern Brazil, has been the subject of ethnopharmacological studies reporting the use of its aerial parts by the local population in the form of cocktail for treatment of gastrointestinal (GIT) diseases (Almeida *et al.*, 2005; 2006). However, there have been few pharmacological studies regarding the safety or efficacy for the species (Agra *et al.*, 2008). Silva et al (2012; 2013) reported on the efficacy and possible mechanism of action of the alcoholic extract of *C. martii* in an experimental model of indomethacin- or ethanol-induced ulcers.

The phytochemical profile of the genus *Chresta* contains predominantly terpenes, including sesquiterpene lactones and flavonoids (Silva *et al*., 2013; Borella *et al*., 1998). The sesquiterpene lactones are lipophilic with bitter taste and colorless, with a broad spectrum of biological activity including cytotoxic, antitumor, antibacterial, anti-inflammatory, schistosomicide, antifungal, and antimalarial. Ghantous *et al*. (2010) and Zhang *et al*. (2005) found that some sesquiterpene lactones were considered highly toxic. In contrast, flavonoids comprise one of the largest groups of secondary metabolites present in the plant kingdom and are noted for their diverse pharmacological properties including anti-tumor, anti-inflammatory, antioxidant, antiviral, antifungicidal and anti-protozoan as well as play a role in reducing the risk of cardiovascular disease (Magalhães *et al*., 2000; Alavez-Solano *et al*., 2000). Since the toxic potential of *C.martii* is not known, this study aimed to determine the acute toxicity *in vivo* and cytotoxicity *in vitro* of organic extracts of *C. martii* as well as genotoxicity *in vivo* of its semi-purified fraction.

MATERIALS AND METHODS

Botanical material

Botanical material of *Chresta martii* (DC.) H. Rob. was collected in the Xingó region, in Sergipe, Brazil, at coordinates ranging from -9.5563° to -9.5548° Latitude and 37.94° Longitude, at an altitude of 130 m, in January 2011. Samples of the material collected were identified by Dr. Nádia Roque (Biology Institute in the Department of Botany, Federal University of Bahia, Brazil) and dried specimens were deposited in the Herbarium HUVA (Sobral, Ceará, Brazil) under number 14602.

Preparation of organic extracts and semi-purified fractions

The preparation of organic extracts involved use of aerial parts of *C. martii* dried in aconvection oven at 50°C and ground in a cutting mill. Subsequently, the material (500 g) was submitted to extraction by maceration at room temperature using organic solvents of increasing polarity: cyclohexane to obtain the cyclohexane extract of *C. martii* (ECCm), ethyl acetate to obtain the ethyl acetate extract of *C. martii* (EACm) and 96% ethanol to obtain the ethanol extract of *C. martii* (EECm) at a proportion of approximately 10g/100 ml solvent. Each extraction cycle lasted 48 hr, using the residue from the first to perform the next extraction, with three replicates for each solvent (Handa *et al.*, 2008). The extracts were concentrated in a rotary evaporator with reduced pressure at 50°C and 90 rpm and/or lyophilized. After determination of yield, samples were stored in amber vials under a temperature of -20°C in a freezer. The EACm (10 g) was fractionated, yielding 4 semi-purified fractions (FI – F4), using a silica gel chromatography column (Vetec 0.063-0.20 mm), eluted with chloroform [Fraction I – F1; yield (10%)], chloroform/ethyl acetate (1/1) [Fraction II – F2; yield (6%)], ethyl acetate [Fraction III – F3; yield (8%)] and ethyl acetate/methanol (1/1) [Fraction IV – F4; yield (5%)]. The semi-purified fractions were concentrated in a rotary evaporator with reduced pressure at 50°C and 90rpm and stored in amber vials at a temperature of -20°C in a freezer.

Phytochemical screening

Qualitative assessment to determine the presence or absence of secondary metabolites in all three extracts of *C. martii* (ECCm, EACm and EECm) was performed using general reactions (Costa, 1982): flavonoids (Shinoda reaction), tannins (reaction with ferric chloride),

terpenoids and steroids (Liberman-Buchard reaction), saponins (foam index) and alkaloids (Dragendorf and Mayer reaction). The quantitative evaluation was utilized to determine total phenolics and total flavonoids according to Swain and Hills (1959) and Woisky and Salatina (1998). The three extracts were further analyzed by High Performance Liquid Chromatography (HPLC) using the same chromatographic conditions as adopted by Silva *et al*., (2013) in order to track the compound isolated, identifying it as a sesquiterpene lactone. To characterize the semi-purified fractions Thin Layer Chromatography (TLC) was used, supported by silica gel 60 (Alugram® Sil G/UV254 produced by Machere-Nagel), eluent system; chloroform: ethyl acetate (8:2 v/v) and anisaldehyde/sulfuric acid as a developer followed by heating (Wagner and Bladt, 1995).

Animals

For determination of acute toxicity, 72 female non-isogenic, nulliparous Swiss Webster mice weighing 25-30 g were used (purchased from the animal facility of the Keizo Asami Laboratory of Immunopathology (LIKA - UFPE). For the assessment of genotoxicity, 15 male non-isogenic Swiss Webster mice weighing 25-30 g were used obtained from the animal facility of the Aggeu Magalhães Research Center – FIOCRUZ-PE, Brazil. All animals underwent adaptation 72 hr before each test and kept under standard conditions (temperature 22 ± 2°C, relative humidity of 30-70% with controlled 12 hr light/dark cycle) according to GLP (Brito, 1994). Animals had access to commercial food (Purina Presence®) and water *ad libitum* before the experiments. The experimental protocols were approved by the Ethics Committee on Animal Use (CEUA) of the Aggeu Magalhães Research Center / FIOCRUZ PE Brazil under the protocol 35/2012 (expiration on 31/01/2017).

Acute toxicity *in vivo*

Acute toxicity by oral (p.o.) and intraperitoneal (i.p.) routes was performed using female Swiss Webster mice (n=6 animals/group). Mice were fasted for 12 hr and weighed before being treated with either of the three organic extracts of *C. martii* at 50, 100, 300, 1000 or 2000 mg/kg. The control group received distilled water (5 ml/kg; p.o. or i.p.). Due to mortality of 2-6 animals by the 14th day, a lower dose was administered subsequently to allow an estimate of a range of LD50, according to the Globally Harmonized System standards (GHS) (OECD- 423/2001).

The animals were assessed for systemic behavior as evidenced by parameters such as general activity, vocal fremitus, irritability, touch response, response to tail compression, contortion, posture of the hindquarters, postural reflex, body tone, grip strength, ataxia, tremors, convulsions, hypnosis, anesthesia, lacrimation, ptosis, urination, defecation, piloerection, breathing, cyanosis and death during the first three hr after administration of the extracts and, from then on, every day, until the 14th day (Malone and Robichaud, 1962; Malone, 1977). Surviving animals were weighed on the 7th and 14th days and anesthetized on the last day with ketamine-xylazine solution of 0.2 ml/100 g (8.75 ml ketamine (100 mg/ml) and 1.25 ml xylazine (100 mg/ml), according to the protocol of Cornell University/Cornell Center for Animal Resources and Education as described by Flecknell (2009) and Kohn *et al.* (1997), and sacrificed by cervical dislocation. A macroscopic analysis was performed on the organs in the thoracic and abdominal cavities and then liver, kidneys and spleen were weighed to determine relative weights (organ weight per 100 g of body weight).

Cytotoxicity assay

The following human cancer cell lines were used: HL-60 (human promyelocytic leukemia), NCI-H292 (human lung carcinoma), and MCF-7 (breast cancer) obtained from the cell bank of Rio de Janeiro (Brazil). The NCI-H292, and MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO2. The HL-60 cells were cultured in RPMI-1640 medium (the formulation is based on the RPMI-1630 series of media utilizing a bicarbonate buffering system and alterations in the amounts of amino acids and vitamins developed by Moore *et al.,* (1963) under the same conditions. The cytotoxicity of the organic extracts was tested using the MTT salt reduction assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma®). For all experiments, tumor cells were plated in 96-well plates (105 cells/ml for each attached cell or 3 x 105 cells/ml for leukemia). Organic extracts (50µg/ml) were dissolved in 1% DMSO and added to each well and incubated for 72 hr. After 69 hr treatment 25 µl MTT (5 mg/ml) was added, three hr later MTT formazan product was dissolved in 100µl DMSO and absorbance read at 595nm in a plate spectrophotometer (Mosmann, 1983).

Micronucleus (MN) test in bone marrow cells

Swiss Webster mice were divided into three groups (n=5 animals/group). The first group was treated with the semi-purified fraction I of *C. martii* (50 mg/kg, i.p.), with the other two groups being controls: the positive one was cyclophosphamide (25 mg/kg, i.p.) because it is an antineoplastic agent with alkylating properties forming a complex with DNA irrespective of whether it is normal or cancer cells (Kijima *et al*., 2003) and the negative one was vehicle [1% carboxymethylcellulose (10ml/kg, i.p.)] of the dilution of fraction I.

The test is characterized by determining acute effects of the chemical agent on anucleated, polychromatic erythrocytes (PCE), as these have a relatively short life, such that any micronucleus (MN) that is present was generated as a result of recently induced chromosome damage (Ribeiro *et al.,* 2003). The positive control used was cyclophosphamide. All animals were sacrificed by cervical dislocation 24 hr post-treatment. Immediately after sacrifice, femurs were excised, dissected and cut at the proximal epiphyses. FBS (1 ml/femur) was injected with a syringe (3 ml) and needle (tuberculin type) into the medullary canal of the femur in order to remove the medullary content. The lavage fluid was collected in a test tube containing 2 ml FBS. The cell suspension was homogenized several times and then centrifuged at 800g for 5 min. The supernatant (3 ml) was discarded, leaving approximately 1 ml for swabbing. The pellet was resuspended and 2 or 3 drops were used to make smears on two, clean and dried slides by sliding one over the other. After leaving the slides for 60 min to dry at room temperature, the material was fixed with methanol (250 ml for 10 min) and stained 24 hr with Leishman's methylene blue dye (Schmid, 1975).

To evaluate the genotoxic (clastogenic) and/or aneugenic potential, 2000 polychromatic erythrocytes (PCE) were analyzed per animal and the frequency of micronucleated PCE (MNPCE) recorded. The cytotoxic effects were investigated by determining PCE-NCE (normochromatic erythrocyte) ratio after analyzing 200erythrocytes/animal. All cells were observed and quantified using an optical microscope (10 x 100x) magnification (Schmid, 1975).

Statistical analysis

The results were expressed as mean ± standard deviation following statistical analyses relevant for each biological test. The occurrence of MN in the groups was compared statistically using the conditional test to compare proportions in situations of rare events (Choy, 2001). Comparisons between groups for the PCE/NCE ratio were performed using

analysis of variance (ANOVA) for completely randomized experiments followed by *Bonferroni*'s post-test with a significance level of 95% ($p<0.05$). All statistical analyses were performed using GraphPad Prism program, version 5.0 for Windows (GraphPad Software, San Diego, California USA).

RESULTS AND DISCUSSION

Extract yield

The yield of the extracts resulted in a decreasing % from 19% for ECCM, 10% for EACm and 5% for EECm. Andreo and Jorge (2006) noted that there is no ideal system for extraction of all or any specific class of natural products due to several factors. Among such factors are the nature of the chemical, extraction method used, solvent used and quantitative difference of these compounds in plants. However, Simões *et al*., (2004) reported that nonpolar solvents have the ability in general to extract lipophilic substances such as waxes, pigments and hydrocarbons. Solvents with medium polarity extract flavonoids and simple coumarin, while agents of high polarity preferentially extract alkaloids, saponins and tannins.

Phytochemical screening

Phytochemical screening showed that *C. martii* has as its secondary metabolites flavonoids, tannins, terpenoids and steroids. It is worth noting that the solvents used enabled a differentiated extraction of these products in terms of their presence and quantity of each organic extract (Table 1).

Table 1 – Phytochemical screening of organic extracts from the aerial parts of *C. martii* (cyclohexane extract of *C. martii* - ECCm: ethyl acetate extract of *C. martii* - EACm and ethanol extract of *C. martii* - EECm)

Chemical class	Extraction procedure	**ECCm**	**EACm**	**EECm**
Saponins	Foam index	-	-	-
Tannins	Ferric chloride	+	++	+++
Alkaloids	Dragendorf. Mayer	-	++	+
Flavonoids	Shinoda	-	+++	+
Steroids and terpenoids	Liberman-Buchard	+	+	-

(-) Not reactive; (+) Low intensity; (++) Medium intensity; (+++) Strong intensity

Silva *et al.*, (2013) reported the presence of a sesquiterpene lactone in the alcoholic extract of *C. martii*, which is corroborated by results presented here. According to Boudet (2008), secondary metabolites are present in all plant species, usually as diverse mixtures and several studies demonstrated the importance of these metabolites in plant defense. Phenolic compounds such as tannins and flavonoids possess antimicrobial and antioxidant properties (Efraim *et al.*, 2006; Yoshida *et al.*, 2008; Lai et al, 2009; Adesegun *et al.*, 2009; Lopez-Lazaro, 2009).

Quantification of phenolic compounds and total flavonoids enabled detection of the presence of total phenolic compounds in EECm and flavonoids and total phenolic compounds in EACm (Figure 1). Phenolic compounds such as flavonoids and tannins present in the plant were detected. Thus, the reduced amount of flavonoids found in EECm suggests the chemical class present in this extract corresponds to the group of condensed tannins. This result corroborates the findings observed in the phytochemical identification of the stated extract.

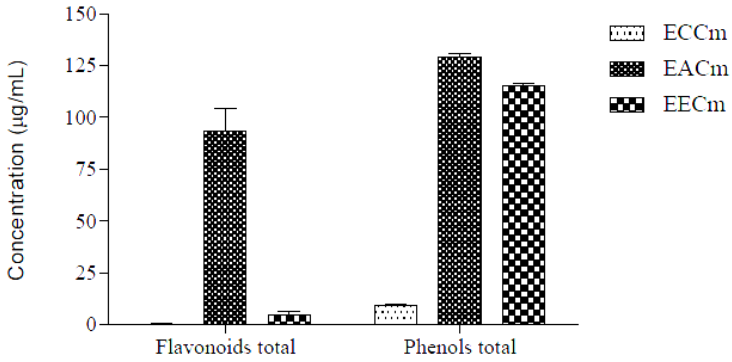

Figure 1 – Quantification of phenols and total flavonoids (through the equation of the line generated from the standard curve of gallic acid (y = 37.583x - 3.8989; R^2 = 0.9977) and of quercetin (y = 75.691x – 140.29; R^2 = 0.9382), respectively). For the organic extracts from the aerial parts of *C. martii* (cyclohexane extract of *C. martii* - ECCm; ethyl acetate extract of *C. martii* - EACm and ethanol extract of *C. martii* - EECm).

The extracts (EACm and EECm) displayed a similar phytochemical profile, a chromatographic peak with the same retention time of ± 15 min and a resolution of 400 and 30 mAU, respectively (Figure 2) was observed with similar characteristics to those found by Silva *et al.*, (2013) and identified as a sesquiterpene lactone. However, no peak with the same retention time was verified for the ECCm extract.

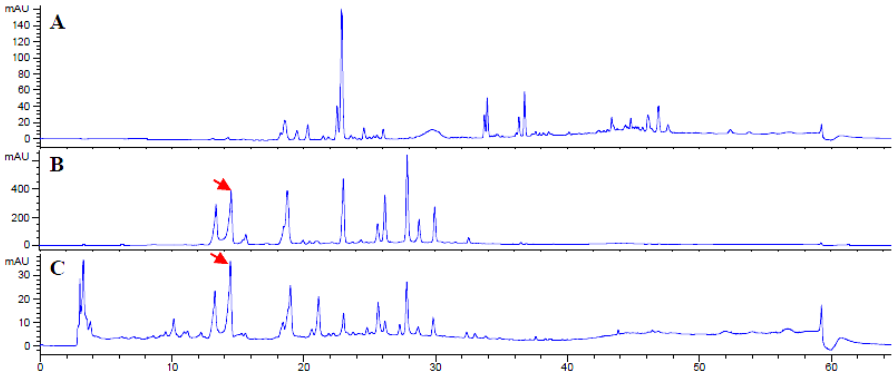

Figure 2 – Chromatogram (HPLC/UV. λ = 254nm) of *C. martii* extracts (ECCm) - A: (EACm) - B: (EECm) - C Red arrows indicate the chromatographic peak (± 15 min retention time) corresponding to compound 1 (sesquiterpene lactone), according to Silva *et al*., (2013).

Acute toxicity

Among the extracts analyzed, ECCm (2000 mg/kg; i.p. or p.o.) was the only one that did not induce behavioral changes worthy of note and did not lead to animal death. Behavioral changes in animals that received EACm and EECm extracts via po or ip administration were observed such as presence of piloerection, tremors, ataxia, reduction of the response to tail compression and depression. These clinical signs varied in intensity, from reversal to death of animals dependent on dose and route of administration.

According to Haggard and Hodges (cited in Leite and Amorim, 2006), compounds that do not exhibit toxicity for LD50 values in the range of 0.5-5 g/kg are classified as slightly toxic. Considering that the LD50 for EACm and EECm were estimated at 500 mg/kg, i.p. or 200 mg/kg, p.o., are thus classified as moderately toxic extracts. Data on mortality observed among the animals treated with each extract are presented in Table 2.

Table 2 – Registration of mortality in groups of female, Swiss Webster mice treated orally or intraperitoneally with organic extracts from the aerial parts of *C. martii* (cyclohexane extract of *C. martii* - ECCm; ethyl acetate extract of *C. martii* - EACm and ethanol extract of *C. martii* - EECm) to estimate the LD_{50} according to the GHS (OECD-423)

Extract	Pathway of administration	Dose (mg/kg)	N° of deaths	estimated LD_{50}
Cyclohexane extract of *C. martii* (ECCm)	Oral	2000	0/6	2500 mg/kg
	Intraperitoneal	2000	0/6	
Ethyl acetate extract of *C. martii* (EACm)	Oral	2000	6/6	500 mg/kg
		1000	4/6	
		300	0/6	
	Intraperitoneal	2000	6/6	200 mg/kg
		1000	5/6	
		300	3/6	
		50	0/6	
96% Ethanol extract of *C. martii* (EECm)	Oral	2000	6/6	500 mg/kg
		1000	2/6	
		300	0/6	
	Intraperitoneal	2000	6/6	200 mg/kg
		1000	4/6	
		300	2/6	
		50	0/6	

Simões *et al*., (2004) and Kabara *et al*., (1972) showed that lipophilic extracts obtained by using highly nonpolar solvents such as petroleum ether, hexane, and cyclohexane are rich in lipids, waxes and pigments have a low rate of acute toxicity, which explains the absence of mortality observed among animals treated with ECCm. However, the solvents used to obtain the extracts EACm and EECm probably extracted terpenoids, including sesquiterpene lactones which according to Zhang *et al*., (2005) are considered highly toxic. The presence of sesquiterpene lactones is striking in the Asteraceae family (Ghantous *et al*., 2010, Herz, 1977). It is worth noting that two sesquiterpene lactones were isolated from the aerial parts of *C. martii* (Silva *et al*., 2013).

Amos *et al*., (2003) treated rodents with artemisinin (50-100 mg/kg; i.p.), a sesquiterpene lactone obtained from the leaves of *Artemisia annua* L. (Asteraceae) and found that this substance appears to act as an antagonist to the post-synaptic D2 receptors in the central nervous system (CNS), thereby reducing exploratory activity and exerting a soothing and sedative effect. It is of interest that plant species containing terpene derivatives have been used as sedatives, tranquilizers and anticonvulsants in traditional medicine (Sousa *et al*., 2008). All of these factors may explain basis for mortality observed in animals receiving high doses of the extracts EACm or EECm, as well as exacerbation of depressive features at the level of the CNS. The neurological effects observed in our study, especially ataxia, corroborate those of Panossian *et al*., (2005) who reported gait ataxia in a patient who utilized artemisia as an alternative treatment for breast cancer.

At the end of the experiment there was no significant difference observed in weight gain of animals. Similarly, there were no macroscopic changes identified in the organs found in the abdominal or thoracic cavity. The relative liver, spleen and kidney weights revealed no significant changes.

Cytotoxicity assay

The MTT assay showed that among the three extracts from *C. martii* (50 ug/ml), EACm and EECm extracts displayed similar cytotoxicity in HL-60 and NCI-H292 cells, while in MCF-7 cells EACm was slightly more cytotoxic. EACm extract rank order of promoted inhibition in the growth of tumor lines was HL-60, followed by NCI-H292 and MCF-7. The second-most cytotoxic extract was EECm, whose growth inhibition of cell lines

ranked highest was HL-60, followed by NCIH-292 and MCF-7. However, the ECCm extract only inhibited growth of HL-60 and MCF-7 lines (Figure 3).

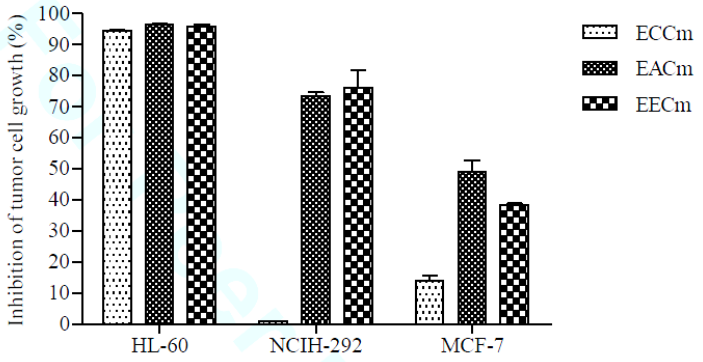

Figure 3 - Percentage growth inhibition of tumor cells HL-60 (human promyelocytic leukemia), NCIH-292 (human lung carcinoma) and MCF-7 (breast carcinoma) exposed to different organic extracts of *C. martii*: cyclohexane extract (ECCm), ethyl acetate extract (EACm) and ethanol extract (EECm), all 50 mg/mL.

Despite these results regarding cell growth inhibition of above 50% for all three tumor cell lines, the American National Cancer Institute has defined the limit for considering an organic plant extract promising for further purification at the threshold of inhibiting cell proliferation to be more than 75% (Suffness and Pezzuto, 1990; Ferreira *et al.*, 2011). However, as this is the first study to show cytotoxicity of extracts of *C. martii* these results deserve attention, especially because it is a species widely used by the population of the region in which it was collected. Although the results of tests that evaluate *in vitro* cytotoxicity may not have a direct correlation with *in vivo* tests, it is safe to say that if a material has been proven to induce a cytotoxic reaction in cell culture it is likely to develop toxicity when applied to living organisms (Osório *et al.*, 1998). Further, for the cell line HL-60, all extracts showed activity greater than 90% for cell growth inhibition. As for the *in vitro* cytotoxic effect observed, this may be linked to the presence of phenolic compounds in the extracts, since Naasani *et al.*, (1998) and Chakraborty *et al.*, (2006) found that phenolic compounds inhibit telomerase activity in tumor cells, inducing death. In addition, Schinor *et al.*, (2004) reported on the growing interest in triterpenoids for their activity (antimicrobial, cytotoxic, analgesic, carcinogenic and allergenic) and indicated the wide occurrence of this compound in the genus *Chresta*. Osorio *et al.*, (1998) suggested that, for the improvement of *in vitro* cytotoxicity testing, the target organ from which the studied cells originated needs to be taken into account. In this study, due to the reduction in the growth of cell line HL-60 by

EACm, the extract was subjected to chromatographic fractionation and the purest product was tested for genotoxic effects *in vivo* in bone marrow cells from Swiss Webster mice.

Chromatographic fractionation of EACm

Four chromatographic fractions were initially obtained from EACm, all characterized by TLC (Figure 4A and 4B). Among them, fraction I (F1) was the purest as determined by its less intense tail throughout the run and its presentation of a single spot when exposed to UV 254 (Figure 4A). Further, when subsequently subjected to anisaldehyde/sulfuric acid, the presence of two spots of purple and lilac color (Figure 4B) was verified that are compatible with compounds of the class of terpenes and steroids, respectively (Wagner and Bladt, 1995).

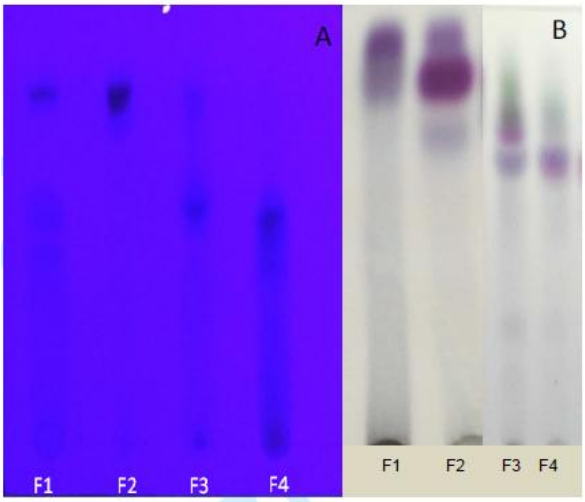

Figure 4 – Characterization of the phytochemical compounds from the semi-purified fractions derived from EACm by TLC and revealed by UV 254 (A) and anisaldehyde/sulfuric acid (B).

Micronucleus (MN) test in bone marrow

The *in vivo* MN test in bone marrow of rodents (Ribeiro *et al.*, 2003) meets the requirements of national agencies such as the National Health Surveillance Agency (ANVISA), which governs the records of pharmacological and chemical products, and also follows the recommendations of the Gene-Tox Program of the Environmental Protection Agency (Micronucleus Test, 2002). The result of the genotoxicity observed in the group treated with fraction I indicated the presence (66.67%) of micronucleated polychromatic erythrocytes (MNPCE) in 10,000 compiled polychromatic erythrocytes (PCE), a value statistically similar to that found in the cyclophosphamide group (positive control) (51.52%).

The group treated with 1% carboxymethylcellulose (negative control) maintained the expected frequency for the physiological pattern of spontaneous induction (3/1000) (Figure 5).

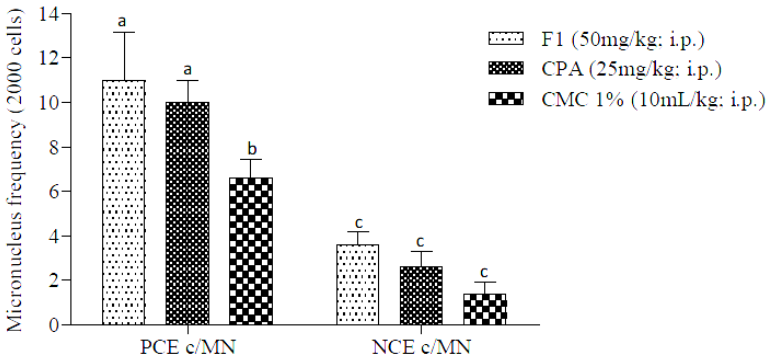

Figure 5 - Micronucleated polychromatic erythrocytes (PCEs) in bone marrow cells of mice treated with: semi-purified *C. martii* fraction I (F1), cyclophosphamide - CPA (25 mg/kg, i.p.) or vehicle (10 ml/kg: i.p.). Columns followed by different letters ($p < 0.05$).

Cytotoxicity was determined by the PCE/NCE ratio for each group. Despite the evident genotoxic effect, cytotoxicity was not observed *in vivo* with administration of fraction I. According to Chacon *et al*., (2002), compounds derived from plant extracts may produce irreversible damage to health leading to death by the action of mutagenic and carcinogenic natural agents. This fact justifies the interest in evaluating *in vivo* mutagenicity due to exposure to the product derived from the *C. martii* extract, principally because it is a species widely used by a particular community, and linked to its culture, for which there does not exist scientific information regarding its genotoxic effects.

CONCLUSIONS

The LD50 estimated for EACm and EECm allowed them to be classified as mild to moderate toxic when administered p.o. or i.p. The compounds present in the extracts of middle and high polarity belong predominantly to the class of phenols and terpenes. It is this phytochemical class that appears to exert *in vitro* cytotoxic activity against human tumor cells. The semi-purified fraction I rich in terpenes and steroids was found capable of inducing DNA damage in mammalian cells as detected by the presence of MN in PCE. In general, *C. martii* is a species with high pharmacological potential and its ideal dosage and administration needs to be established to avoid possible adverse effects.

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4.1.2 ARTIGO SUBMETIDO À PUBLICAÇÃO PELO *EVIDENCE-BASED COMPLEMENTARY AND ALTERNATIVE MEDICINE*

Fator de impacto: 2.175; Qualis CAPES área de Biotecnologia: A1

Study of the gastroprotective effect of extracts and semi-purified fractions of *Chresta martii* DC. and identification of its principal compounds

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Chresta martii (Asteraceae) is a species widely used by the population of the Xingó region - Sergipe, Brazil, in the form of a decoction (aerial parts) for the treatment of gastrointestinal diseases. The study aimed to assess the gastroprotective activity of organic extracts and semi-purified fractions and identify the principal compounds present in *C. martii* responsible for such activity. The organic extracts (cyclohexane – ECCm, ethyl acetate – EACm and ethanol – EECm) were obtained from the dried aerial parts (500 g) of *C. martii*. For evaluation of the gastroprotective activity of extracts (50, 100 or 200 mg/kg; v.o.) male *Swiss Webster* mice (25-30 g) were used which had gastric ulcers induced by indomethacin (40 mg/kg, s.c.) or ethanol (0.2 mL/animal; v.o.). Among the extracts evaluated, EACm exhibited significant ($p < 0.05$) gastroprotective activity in the models used. The fractionation of EACm was performed in a silica gel column 60 eluded with the following compounds: [chloroform-F1 yield (10%)], [chloroform/ethyl acetate (1/1) - F2 yield 6%)], [ethyl acetate - F3 yield (8%)] and [ethyl/methanol acetate (1/1) - F4 yield (5%)]. Of the fractions described above, the F1 (25 mg/kg; v.o.) had the greater gastroprotective activity ($p<0.05$) than that displayed by ranitidine (80 mg/kg; v.o.) in the ethanol-induced ulcer model. The refractionation of F1

produced 23 subfractions and from these two yellow amorphous compounds were obtained by recrystallization, Rf: 0.46 and 0.31 (ethyl acetate; chloroform 5:5). The compounds isolated were characterized by nuclear magnetic resonance spectroscopy (1H-NMR and 13C-NMR) and identified as flavones: chrysoeriol (yield - 0.43%) and 3',4'-dimethyloxiluteolin (yield - 0.58%). Conclusion: flavone 3',4'-dimethyloxiluteolin is the principal compound present in the species *C. martii* and is probably be responsible for gastroprotective activity observed in this species.

Keywords: gastroprotectant, antioxidant, 3',4'-dimethyloxiluteolin, *Chresta martii*, mice

INTRODUCTION

Gastric and duodenal ulcers are associated with the imbalance between the protective and aggressive factors of the gastric mucosa (De-Sousa *et al*., 2008). Among the protective factors are prostaglandins, nitric oxide, mucus and bicarbonate (Araujo *et al*., 2002; Donatini *et al*., 2009). However, the aggressive factors which predispose the emergence of gastric ulcers are the following: non-steroidal anti-inflammatory medications (NSAIDs), *Helicobacter pylori* infections and the chronic use of alcoholic beverages (Almeida *et al*., 2012; Ddine *et al*., 2012). It is known that this imbalance can significantly impact control of hydrochloric acid secretion by the stomach, whose production has hormonal and neural influences. Hormonal control (endocrine and paracrine) occurs in a complex manner via at least three different cell types: enterochromaffin-like cells producing histamine, G cells producing gastrin; and D cells secreting somatostatin. Neural control is linked to vagal cholinergic control, which acts on all these cells and parietal cells. Histamine, gastrin and acetylcholine, the latter released by the vagus, directly and positively influence the production of HCl by parietal cells. Somatostatin acts in reverse, inhibiting the release of histamine and gastrin and, consequently, the production of acid (Tobin *et al*., 2009).

This pathology is the most common chronic illness among adults, affecting 5 to 10% of the world's population. In children, from four to seven new cases of gastric ulcers are registered per year in the major pediatric centers (Carvalho, 2000). In both cases the treatment is carried out using drugs which act by inhibiting the proton pump, neutralizing the acid secretion (antacids) or blocking the histamine receptors (H2 antihistamines) (Mulholland and Debas, 1987). Although these medications can produce serious adverse reactions, including hypersensitivity, arrhythmia, and impotence (Malfertheiner *et al*., 2009), they are commonly used for the treatment of gastric ulcers (Ddine *et al*., 2012). From this perspective, investigations into the use of medicinal plants is supported by the richness of the Brazilian

flora, and the search for new anti-ulcerogenic agents, most notably with regard to the identification of their principal compounds, elucidation of structure and pharmacological activity (safety and efficacy) have all been the target of constant research (BRASIL, 2011).

Chresta martii (Asteraceae) is popularly known as *muricica*. In the Xingó region - Sergipe, Brazil, this species is widely used in the form of a decoction of aerial parts (±10g/L) for the treatment of gastrointestinal diseases (Almeida *et al*., 2005a, 2006b). Until now only two studies, using an experimental model of gastric ulcers, have been published reporting the gastroprotective activity of a hydroalcoholic extract of *C. martii*. One of the studies identified a compound belonging to the class of terpenes (sesquiterpene lactone) (Silva *et al*., 2012, 2013). Thus, the present study aimed to investigate the gastroprotective activity of different organic extracts and semi-purified fractions of *C. martii* in experimental models of gastric ulcers induced by indomethacin or ethanol in *Swiss Webster* mice and identify the class and chemical structure of the principal compounds coming from the product with the best gastroprotective activity.

MATERIALS AND METHODS

Botanical material

Chresta martii (DC.) H. Rob. was collected in the Xingó region located in Sergipe, Brazil at coordinates ranging from -9.5563 to -9.5548 latitude, 37.940 longitude and an altitude of 130 meters) in January 2011. Samples of the material collected were identified by Dr. Nádia Roque (Institute of Biology in the Department of Botany at the Federal University of Bahia, Brazil) and plant specimens were deposited at the herbarium HUVA (Sobral, Ceará, Brazil) under number 14602.

Animal model

Male *Swiss Webster* mice (25-30 g) were kept at 22 ± 2°C under a light/dark cycle of 12/12 h, with water and food *ad libitum*. Before the experiment (24 h) the animals were fed a liquid diet (5% glucose solution) and water *ad libitum*. All treatments and protocols were carried out according to the "Practical Manual on the Ethical Use and Care of Laboratory Animals" of the Brazilian Society of Science in Laboratory Animals (SBCAL). The experiments are in accordance with the Guidelines for the Care and Use of Laboratory Animals. The research was approved and licensed (# 23076.015207/2012-42) by the

Commission of Ethics in the Use of Animals (CEUA) of the Federal University of Pernambuco, Brazil.

Preparation of organic extracts and semi-purified fractions

To obtain the organic extracts, aerial parts of *C. martii* were used that had been dried in a convection oven at 50ºC and pulverized in a knife mill. Subsequently, the material (500 g) was submitted to extraction by maceration at room temperature using organic solvents of increasing polarity: cyclohexane (ECCm), ethyl acetate (EACm) and 96% ethanol (EECm), at a proportion of approximately 10 g /100 mL of solvent. Each extraction cycle lasted 48 hours, using the residue of the former to perform the next extraction, with three repetitions for each solvent (Singh, 2008). The extracts were concentrated in a rotary evaporator with reduced pressure at 50ºC and 90 RPM and/or lyophilized. After the determination of yield, the extracts were stored in amber bottles and kept at a temperature of -20°C (freezer) until the moment of their use, at which time the material was diluted in saline (0.9% NaCl).

The EACm extract (10 g) was submitted to fractionation, giving rise to four semi-purified fractions (FI – F4), using silica gel column chromatography (Vetec 0.063-0.20 mm), eluded with chloroform [Fraction I – F1; yield (10%)], chloroform/ethyl acetate (1:1) [Fraction II – F2; yield (6%)], ethyl acetate [Fraction III – F3; yield (8%)] and ethyl acetate/methanol (1:1) [Fraction IV – F4; yield (5%)]. The semi-purified fractions were concentrated in a rotary evaporator with reduced pressure at 50ºC and 90 RPM and stored in amber bottles, at a temperature of -20° C (freezer) until the moment of its use, at which time the material was diluted in a saline solution (0.9% NaCl).

Isolation and identification of the principal compound(s) of fraction (F1)

The F1 fraction (3 g) underwent refractionation using silica gel column chromatography (Vetec 0.063-0.20 mm), eluded with chloroform/ethyl acetate initially (9/1), with a gradual increase in polarity. This procedure resulted in a total of 23 subfractions arising from the mixing of similar samples as verified by thin-layer chromatography (TLC) - silica gel 60 F254 (pre-coated sheets of aluminum - Macharey Nagel), revealed with anisaldehyde/sulfuric acid. The subfractions were dried at room temperature for crystallization of the compounds. Then the residue was resuspended in acetone for recrystallization (Costa, 1994) and filtered to obtain pure crystals. These crystals were identified by nuclear magnetic resonance spectroscopy (NMR).

Magnetic Resonance Imaging (MRI)

The spectra of ^{1}H and ^{13}C-NMR were recorded using the Varian Unity Plus model, 300 MHz for ^{1}H and 75 MHz for ^{13}C in the Analytical Center of the Fundamental Chemistry Department – UFPE. The chemical shifts (δ) were reported in ppm and the coupling constants in Hertz (Hz). Tetramethylsilane (TMS) was used as an internal reference standard. The crystals were solubilized in DMSO-*d*6.

Study of the gastroprotective effect of the organic extracts and semi-purified fraction of *C. martii*

Gastric lesions induced by indomethacin

Gastric ulcerations were induced in *Swiss Webster* mice (n=6 animals per group) held under fasting (24 h) by the administration of indomethacin (Sigma®) (40 mg/kg; s.c.). The animals were pre-treated with extracts (ECCm, EACm or EECm) each in doses of 50, 100 or 200 mg/kg (v.o.), with a positive control of omeprazole (30 mg/kg; v.o.) or a negative control of saline (5 mL/kg; v.o.), 1 h before the administration of indomethacin. A group containing mice (n=3) without ulcer induction received only saline (5 mL/kg; v.o.) to be used as the default physiological group. The doses of extracts were chosen taking into consideration the prior determination of LD50 (results not presented) and by comparison with the dose used by Silva *et al*., (2012 and 2013) for the same plant species and model of ulcer. The animals were euthanized six hours after the ulcerogenic procedure. Their stomachs were removed and opened along the greater curvature. The ulcer index was evaluated using the quantitative method for gauging the extent of erosion and experimental gastric ulcers as described by Szabo *et al*. (1985). The percentage of inhibition was calculated in relation to the saline group according to the following formula: % inhibition = UIt/UIs × 100, where UIt and UIs correspond to the ulcer indices of the treated and saline groups, respectively.

Ethanol-induced gastropathy

The gastric damage was induced by the administration of 99.9% ethanol (0.2 mL/animal) in *Swiss Webster* mice (n=6 animals per group) kept under fasting (24 h) (Morimoto *et al*., 1991). The animals were treated an hour before induction with EACm (50, 100 or 200 mg/kg; v.o.), or semi-purified fractions F1, F2, F3, F4 (50 mg/kg; v.o.) or only F1 (12.5, 25 or 50 mg/kg; v.o.). The control groups were treated with ranitidine (80 mg/kg; v.o.) as a positive control or saline (5 mL/kg; v.o.) as a negative control. A group containing mice

(n=3) without ulcer induction received saline (5 mL/kg; v.o.) to be used as a physiological parameter group. All the animals were euthanized in a CO2 chamber 30 min after the injurious procedure. Their stomachs were removed and opened along the greater curvature, washed in saline, fixed between petri dishes and photographed (Sony Cyber-shot Dsc-h2) at 72 dpi resolution (2816 × 2112 pixels). Hemorrhagic or ulcerative lesions were measured and compared to the total area of each stomach through computerized planimetry using the Image J program (National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland, USA). The percentage of inhibition was calculated in relation to the saline group according to the following formula: %inhibition = UIt/UIs × 100, where UIt and UIs correspond to the indices of ulcers in the treated and saline groups respectively (Morimoto *et al*., 1991).

Statistical analysis

All values were expressed as mean ± SD. For the ulcerative/hemorrhagic area, ANOVA followed by the *Bonferroni* test for multiple comparisons were used. The differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

The gastroprotective effect of organic extracts (ECCm, EACm and EECm) on gastric lesions induced by indomethacin is illustrated in Figure 1.

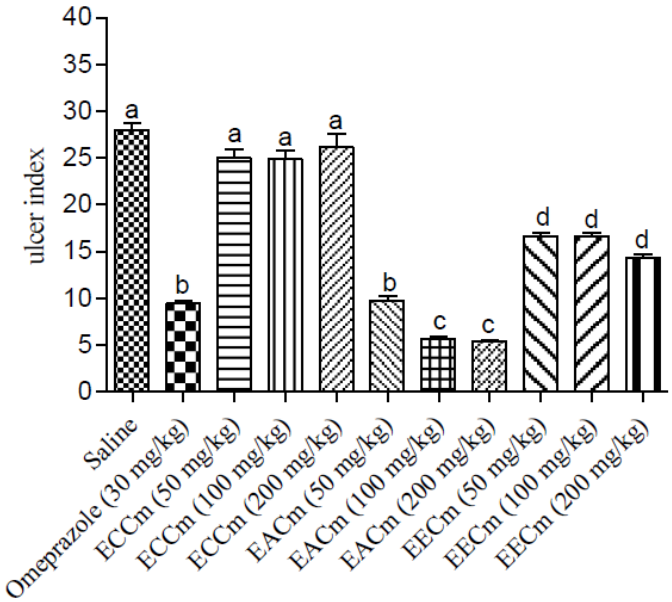

Figure 1 – Gastroprotective effect of cyclohexane extract of *C. martii* (ECCm); ethyl acetate extract of *C. martii* (EACm) and ethanolic extract of *C. martii* (EECm) at doses of (50, 100 or 200 mg/kg; v.o.) on gastric lesions induced by indomethacin in mice (n=6). Different letters indicate minimal significance ($p < 0.05$) *Bonferroni* test.

As can be observed, the EACm was the extract which conferred the best gastroprotective activity. The activity observed in the group treated with EACm (50 mg/kg) was significantly ($p < 0.05$) different from the control group (saline) and comparable ($p > 0.05$) to that observed in the group treated with omeprazole (30 mg/kg; v.o.). A previous study using a hydroalcoholic extract of aerial parts of *C. martii* (100 or 400 mg/kg; v.o.) showed significant gastroprotective activity in an ulcer model induced by indomethacin, with inhibition indices of 54.66 and 81.30, respectively (Silva *et al*., 2013). Our results demonstrate superior gastroprotective activity with EACm (100 or 200 mg/kg; v.o.) (79.89% and 80.83%, respectively) taking into consideration that the second dose of EACm was 50% less than the highest dose used in Silva *et al*., (2013).

The best performance observed in our work was due to the extractive process and choice of solvent. These two factors favored the best extraction of substances present in *C. martii*, resulting in significant gastroprotective activity. The ability of indomethacin to induce ulcers experimentally and in humans has been well documented in the literature (Borrelli and Izzo, 2000), because it is a potent inhibitor of cyclooxygenases, preventing the biosynthesis of prostaglandins (Vane, 1971). The reduction in biosynthesis of prostanoids would result in decreased resistance of the gastric mucosa to the action of HCl and pepsin as it induces an imbalance between the protective and aggressive factors of the mucosa (De-Sousa *et al*., 2008), taking into account the previous knowledge of the mechanism of action of indomethacin on the gastric mucosa. The likely mechanism of action assigned to EACm can be deduced to involve the biosynthesis of prostaglandins or mimetic action of the same as occurs with synthetic analogs of PGE1 (e.g. misoprostol). This mimetic effect can act through stimulation of secretion of mucus, bicarbonate and/or increased blood flow to the stomach.

Similar to what was verified with the model of gastric injury induced by indomethacin, EACm (50 mg/kg; v.o.) also presented a cytoprotective effect against ethanol-induced gastric lesions. The gastroprotective effect was dose-dependent and, in all doses tested, superior to that verified with ranitidine (Figure 2).

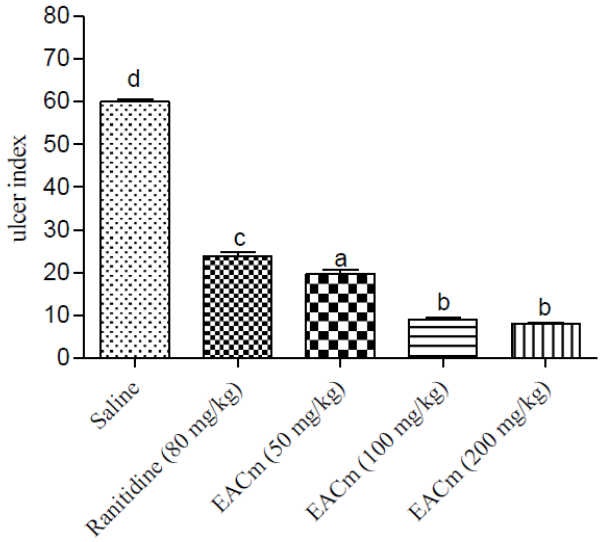

Figure 2 – Gastroprotective effect of an ethyl acetate extract of *C. martii* (EACm) (50, 100 or 200 mg/kg; v.o.) in ethanol-induced gastric lesions in mice (n=6). Different letters indicate minimal significance ($p < 0.05$) *Bonferroni* test.

Ethanol-induced gastric lesions involve different mechanisms such as: a reduction in bicarbonate secretion, a reduction in mucus production, damage to gastric blood flow and direct injury to mucosal cells (Birdane *et al*., 2007; Marhuenda *et al*., 1993). These lesions are associated with the excessive production of free radicals, which attack essential cellular constituents such as nucleic acids, proteins and lipids (La-Casa *et al*., 2000). The increase of the content of lipid peroxides and oxygen-derived free radicals results in significant changes at the cellular level and cause damage to membranes, cell death, exfoliation and epithelial erosion (Birdane *et al*., 2007). Studies have demonstrated the presence of flavonoids in species belonging to the family Asteraceae and this chemical class has been shown to have important antioxidant properties. This could be one of the forms of action of the compounds present in EACm in providing significant cytoprotection, given that free radicals are one of the harmful factors of the gastric mucosa induced by ethanol.

Evaluation of the semi-purified fractions (F1, F2, F3, F4) (50 mg/kg; v.o.) against the ethanol-induced ulcer model revealed that animals treated with F1 and F2 showed lower rates of ulcers when compared to the ranitidine control. However, when compared with each other, F1 presented a significantly lower ulcer index (Figure 3A). Additionally, the gastroprotective effect presented by F1 was dose-dependent (Figure 3B).

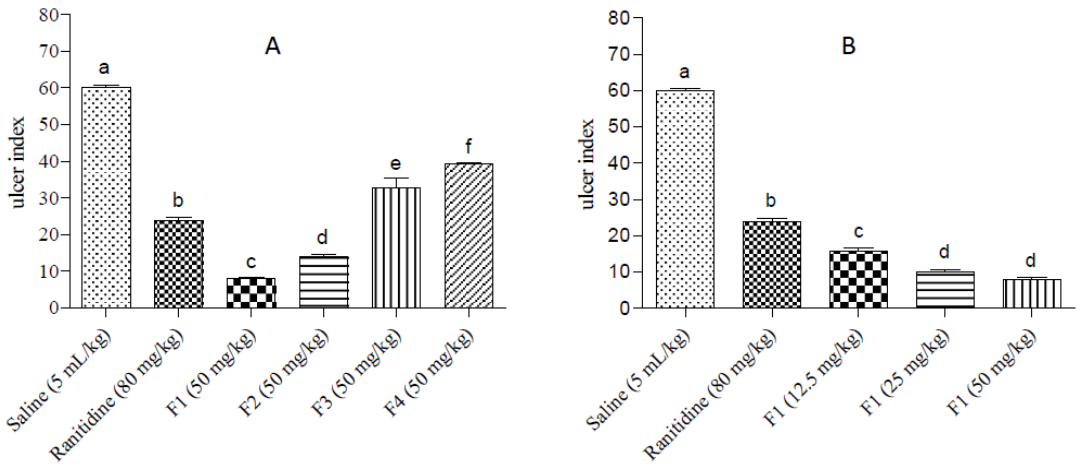

Figure 3 – Gastroprotective effect of fract ions (F1, F2, F3, F4) (50 mg/kg; v.o.) (A) and the fraction (F1) in isolation (50, 25 or 12.5 mg/kg; v.o.) (B) obtained from the ethyl acetate extract of *C. martii* on ethanol-induced gastric lesions in mice (n=6). Different letters indicate minimal significance ($p < 0.05$) *Bonferroni* test.

After verification of the significant gastroprotective activity of F1, it was refractioned, giving rise to 23 subfractions in which the presence of bands was determined through TLC, revealing anisaldehyde/sulfuric acid, characteristics of flavonoids (yellow), steroids (lilac) and terpenoids (blue) (Figure 4). The existence of a larger number of bands with colors ranging from orange to yellow can be seen. Two pure substances F1.1 (Rf: 0.31) and F1.2 (Rf: 0.48) were recrystallized, showing a yellow, amorphous physical appearance.

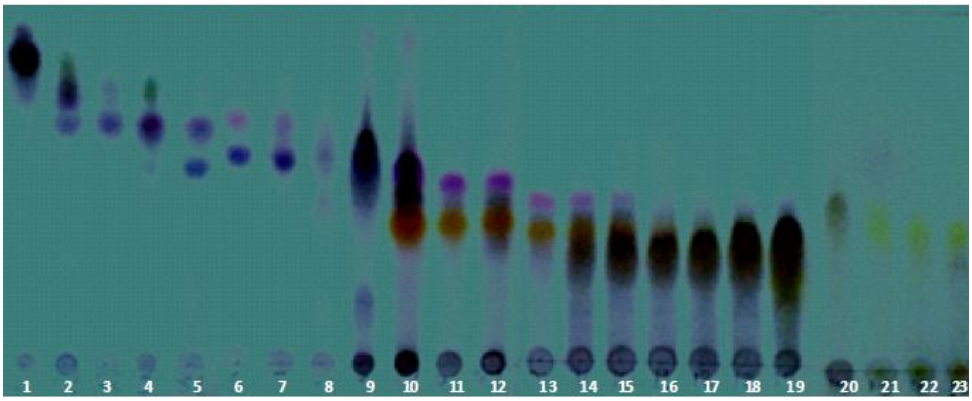

Figure 4 – Chromatographic profile by TLC of the eluded fract ion (F1) (chloroform:ethyl acetate 8:2), revealed with anisaldehyde/sulfuric acid.

The NMR (^{1}H and 13C) presented peaks from which it was possible to calculate the chemical shift (δ) ppm and the coupling constants Hertz (Table 1).

Table 1 – Data of ^{1}H- and ^{13}C- NMR of flavonoids (F1.1 and F1.2) isolated from the F1 of *C. martii* (300 MHz) in DMSO- *d*6

Positions	flavonoid (F1.1) Chrysoeriol		flavonoid (F1.2) 3',4'-Dimethoxyluteolin	
	δ ^{1}H (*J*, Hz)	**δ ^{13}C**	**δ ^{1}H (*J*, Hz)**	**δ ^{13}C**
2		163.7		163.9
3	6.88 (*s*)	104.1	6.97 (*s*)	106.0
4		181.8		181.5
5		162.0		163.0
6	6.19 (*d*. 1.8)	98.0	6.21 (*d*. 2.1)	98.2
7		164.2		164.7
8	6.52 (*d*. 1.8)	94.0	6.53 (*d*. 1.8)	94.0
9		157.4		157.9
10		104.0		104.0
1'		121.0		120.6
2'	7.56 (*d*. 2.4)	110.0	7.56 (*d*. 2.1)	109.8
3'		148.1		148.0
4'		150.8		151.7
5'	6.94 (*dd*. 8.4; 2.4)	116.0	7.13 (*d*. 8.4)	116.2
6'	7.55 (*dd*. 8.4; 2.4)	122.0	7.68 (*dd*. 9.6; 1.8)	121.6
5-OH	12.97			
8-OH	10.79			
4'-OH	9.96			
3'-O$\underline{C}$H$_3$	3.89 (*s*)	56.0	3.85 (*s*)	56.0
4'-O$\underline{C}$H$_3$			3.88 (*s*)	56.0

From the interpretation of the spectra two flavones were identified: chrysoeriol (F1.1) and 3',4'-dimethoxyluteolin (F1.2) (Figure 5 - A and B). The yield of 3',4'-dimethoxyluteolin was 0.58% and was higher than that observed for chrysoeriol at 0.43%. It was verified in this study that the principal compounds present in EACm are flavones.

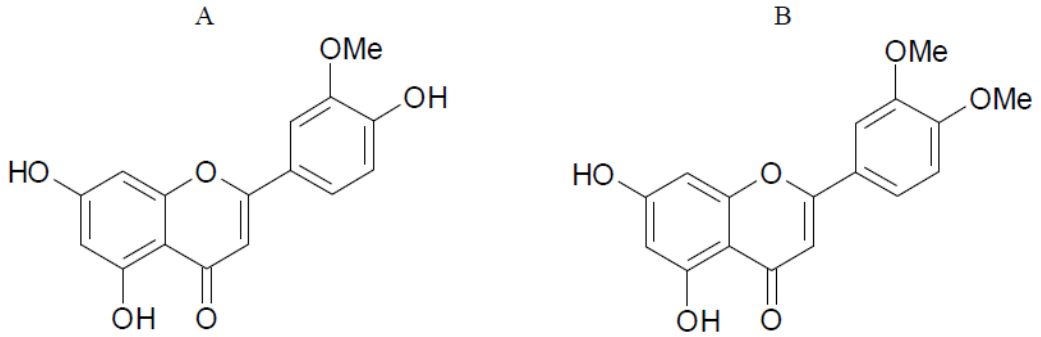

Figure 5 - Chemical structure of chrysoeriol (A) and 3',4'-dimethoxyluteolin (B) isolated from *Chresta martii*

This study is a pioneer in the identification of two flavones of *Chresta martii*. According to Van and Lea (1985), flavones present at least four important physiological

mechanisms: they 1) bind to enzymes in the cell membrane; 2) bind to heavy metal ions; 3) participate in electron transfer of enzymatic systems; 4) sequester free radicals.

The flavone (3',4'-dimethoxyluteolin), among other flavonoids, was assessed as to its gastroprotective and anti-inflammatory potential (in different experimental models of gastric ulcers and inflammation using rodents). The mechanism of gastroprotective action proposed for these compounds has been reported to be the metabolic activation of arachidonic acid via cyclooxygenase activation leading to biosynthesis of prostaglandins, such as PGE2 and PGI2, both with important protective function of the gastric mucosa. The compounds were furthermore able to inhibit the activation of 5-lipoxygenase (leukotrienes), which is an important inflammatory mediator, besides acting in the sequester of free radicals. These results have given rise to some patents by Yoo *et al*. (1998) (EPO 004541), Yoo *et al*. (2000) (US 6025387), Yoo *et al*. (2002) (EPO 0915864B1). Similarly, Sadik *et al*., (2003) verified potent antioxidant and inhibitory activity of lipoxygenase in culture of rabbit reticulocytes when different flavonoids were used including luteolin, whose structure is very similar to 3',4'-dimethoxyluteolin.

However, the fraction (F1) is a mixture (terpenes, flavonoids and steroids) and these compounds can act synergistically with regard the gastroprotective potential presented here, possibly involving other mechanisms of action beyond those cited by Yoo *et al*. (1998a, 2000b, 2002c), such as activation of alpha-2 adrenergic receptors as proposed by Silva *et al*., (2012) when evaluating the hydroalcoholic extract of *C. martii* in an ethanol-induced ulcer model.

There is a scarcity of studies that assess the phytochemical profile, at the level of identifying the principal compounds, for *C. martii*. Such work was pioneered by Silva *et al*., (2012 and 2013) in identifying two sesquiterpene lactones directly from a hydroalcoholic extract. The present study documents for the first time identification of two flavones (3',4'-dimethoxyluteolin and chrysoeriol) from the genus *Chresta*, with relevant evidence for these being the agents responsible for the gastroprotective effect seen in *C. martii*, given that this investigation occurred through direct monitoring between gastroprotective pharmacological activity and processes of extraction, purification and identification of compounds. Finally, the results presented here provide rational support for the purported use of the species in the treatment of gastrointestinal disorders, according to ethnopharmacological information.

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Yoo, M. *et al.*, Gastroprotective flavone/flavanone compounds with therapeutic effect on inflammatory bowel disease. *United States Patent (USP)*, 6025387, 02.15.2000

Yoo, M. *et al.*, Gastroprotective flavone/flavanone compounds with therapeutic effect on inflammatory bowel disease. *European Patent Office (EPO)*, 0915864 B1, 04.09.2002

5 CONSIDERAÇÕES FINAIS

A totalidade dos resultados alcançados com este trabalho possibilitou a comprovação científica da atividade gastroprotetora de *Chresta martii*, fato que respalda a utilização etnofarmacológica dessa espécie pela população do xingó (Sergipe/Brasil);

Contudo, o rastreamento farmacológico e toxicológico dos diferentes extratos demonstrou que *Chresta martii* apresenta no mesmo perfil de polaridade ativos também responsáveis pela gastroproteção, no entanto, tóxicos. Dessa forma, sua administração deve ser criteriosa no que concerne a dose terapêutica;

A identificação química de uma flavona como composto majoritário, com atividade gastroprotetora, é de grande importância haja vista, a maioria dos relatos tratar de sesquiterpenos lactonas, para essa família/gênero, com essa atividade;

A elucidação da estrutura química dos seguintes flavonóides (Chrisoeriol e do 3',4'-Dimetoxiluteolina) possibilitou inferir que essas substâncias além da propalada atividade antioxidante podem apresentar outros mecanismos que possibilitem sua atuação como agente gastroprotetor.

Por fim, com esse trabalho será possível contribuir com a política do uso racional de plantas medicinais através da difusão do conhecimneto e da comprovação científica das propriedades medicinais de *C. martii*, bem como disponibilizar opções inovadoras para o mercado de fitomedicamentos.

ANEXO – A

Parecer da Comissão de ética no Uso de Animais (CEUA) da UFPE

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

Av. Prof. Nelson Chaves, s/n
50670-420 / Recife - PE - Brasil
fones: (55 81) 2126 8840 | 2126 8351
fax: (55 81) 2126 8350
www.ccb.ufpe.br

Recife, 01 de outubro de 2012.

Ofício nº 482/12

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª Maria Bernadete de Sousa Maia**
Departamento de Fisiologia e Farmacologia
Universidade Federal de Pernambuco
Processo nº 23076.015207/2012-42

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Efeito de compostos bioativos de origem vegetal em roedores."**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério do Departamento de Fisiologia e Farmacologia - UFPE; Animais: camundongos e ratos; Sexo: fêmeas e machos; Idade: 45 dias; Peso: 25 - 30g; Número de animais previsto no protocolo: 120 ratos e 180 camundongos.

Atenciosamente,

Profª Marcia Vasconcelos
Vice-Presidente do CEUA/CCB-UFPE
SIAPE 2199635

CCB: Integrar para desenvolver

ANEXO – B

Parecer da Comissão de ética no Uso de Animais do Centro de Pesquisas Aggeu Magalhães/ Fundação Oswaldo Cruz (CEUA/CPqAM)

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado INVESTIGAÇÃO DO POTENCIAL MUTAGÊNICO E/OU GENOTÓXICO DE PRODUTOS NATURAIS, COMPOSTOS QUÍMICOS E BIOLÓGICOS, QUE INTERFIRAM NO ÂMBITO DA SAÚDE HUMANA E MEIO AMBIENTE, ATRAVÉS DE MODELOS EXPERIMENTAIS *IN VIVO* E *IN VITRO*. Protocolado sob nº 35/2012 pelo (a) pesquisador (a) Drª Maria Eliane Bezerra de Mélo. Está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (CEUA/CPqAM) em 30/01/2013. Na presente versão, este projeto está licenciado e tem validade até 31 de janeiro de 2017.

Quantitativo de Animais Aprovados	
Espécie	**Nº de Animais**
Camundongos (Mus musculus) Swiss webster femeas	**400**
Camundongos (Mus musculus) Swiss webster machos	**400**

We certify that project entitled INVESTIGAÇÃO DO POTENCIAL MUTAGÊNICO E/OU GENOTÓXICO DE PRODUTOS NATURAIS, COMPOSTOS QUÍMICOS E BIOLÓGICOS, QUE INTERFIRAM NO ÂMBITO DA SAÚDE HUMANA E MEIO AMBIENTE, ATRAVÉS DE MODELOS EXPERIMENTAIS IN VIVO E IN VITRO. Protocol nº 35/2012), coordinated by Maria Eliane Bezerra de Melo. Is according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz on January, 30, 2013. In present verson this project is licensed and valid until December, 31, 2017.

Recife (PE, Brazil) January, 30, 2013.

DRA. LAURA GE...
Coordenadora da Comissão de Ética no Uso de Animais - CEUA

Drª Laura Helena Vega Gonzalez
Coordenadora CEUA/CPqAM

Av. Professor Moraes Rego, s/n - Cidade Universitária – Campus da UFPE
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ANEXO – C

Carta de aceito do artigo submetido ao Journal of Toxicology and Environmental Health, Part A: Current Issues

Journal of Toxicology and Environmental Health, Part A: Current Issues

Preview

From: sam.kacew@uottawa.ca

To: eryvelton_franco@hotmail.com

CC:

Subject: Journal of Toxicology and Environmental Health, Part A: Current Issues - Decision on Manuscript ID UTEH-2014-0365.R1

Body: 30-Dec-2014

Dear Dr Franco:

Ref: EVALUATION OF THE ACUTE TOXICITY, CYTOTOXICITY AND GENOTOXICITY OF Chresta martii (ASTERACEAE)

Our reviewers have now considered your paper and have recommended publication in Journal of Toxicology and Environmental Health, Part A: Current Issues. We are pleased to accept your paper in its current form which will now be forwarded to the publisher for copy editing and typesetting. The reviewer comments are included at the bottom of this letter.

You will receive proofs for checking, and instructions for transfer of copyright in due course.

The publisher also requests that proofs are checked through the publisher's tracking system and returned within 48 hours of receipt.

Thank you for your contribution to Journal of Toxicology and Environmental Health, Part A: Current Issues and we look forward to receiving further submissions from you.

Sincerely,
Dr Kacew
Editor in Chief, Journal of Toxicology and Environmental Health, Part A: Current Issues
sam.kacew@uottawa.ca

ANEXO – D

Instruções aos autores para submissão de artigo ao Journal of Toxicology and Environmental Health, Part A: Current Issues

Instructions for authors

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Submission of Manuscripts

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ANEXO – E

Comprovante de submissão e avaliação do artigo pelo Evidence-Based Complementary and Alternative Medicine

576495.v1 (Research Article)

Title	Study of the gastroprotective effect of extracts and semi-purified fractions of Chresta martii DC. and identification of its principal compounds
Journal	Evidence-Based Complementary and Alternative Medicine
Issue	Regular
Additional Files	Cover Letter
Manuscript Number	576495 (Research Article)
Submitted On	2014-11-29
Author(s)	Eryvelton de Souza Franco, Maria Eliane Bezerra de Mélo, Bernardo José de A. Jatobá, Andréa Lopes Bandeira Delmiro Santana, Antonio Alfredo Rodrigues e Silva, Teresinha Gonçalves Silva, Márcia Silva do Nascimento, **Maria Bernadete Souza Maia**
Editor	Mahmood A. Abdulla
Status	Under Review

ANEXO – F

Instruções aos autores para submissão de artigo a Evidence-Based Complementary and Alternative Medicine

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Apêndice – A

ARTIGO PUBLICADO NO *J. NAT MED* - 2013

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ORIGINAL PAPER

Protective effect of *Chresta martii* extract against indomethacin-induced gastric lesions in mice

A. A. R. Silva · M. M. Bezerra · H. V. Chaves · K. M. A. Pereira · J. A. Aguiar · V. P. T. Pinto · C. Abbet · C. A. Simões-Pires · E. S. Franco · A. T. Henriques · K. Hostettmann · M. B. S. Maia

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Abstract *Chresta martii* (Asteraceae) is a plant found in the Xingó region (semi-arid area) in Northeastern Brazil, and is recognized by the local population as a traditional herb used to treat gastric diseases. This is the first report of the chemical composition, acute toxicity, and gastroprotective effect in mice of the hydroalcoholic extract (HAE) from the aerial parts (leaves and flowers) of *Chresta martii*. Animals received HAE doses from 10 to 2000 mg/kg, i.p. or 50 to 3000 mg/kg, p.o.) and were observed over 48 h for toxicity signs and mortality; sub-chronic toxicity was evaluated through 14 days treatment with once-daily HAE doses (400 mg/kg, p.o.). The gastroprotective effect of HAE was demonstrated on the indomethacin-induced gastric ulcer model after the administration of extracts. Data comparison of ulcer index averages between saline and HAE (100 or 400 mg/kg, p.o.) groups showed significant ($P < 0.01$) inhibition (71.73 and 76.72 %, respectively) of indomethacin-induced gastric lesions. Histological analyses showed significant ($P < 0.05$) inhibition of leukocyte migration in HAE-treated groups. A fingerprint of the HAE obtained by

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HPLC/UV/MS analysis showed major peaks characteristic of sesquiterpene lactones. Compound **1** was isolated and elucidated as a new natural product. Its capacity to prevent leukocyte chemotaxis was demonstrated in vitro, corroborating the pharmacological effects observed for *C. martii* HAE.

Keywords *Chresta martii* · Asteraceae · Sesquiterpene lactones · Gastric ulcer

Introduction

Despite progress in diagnosis and treatment, peptic ulcer disease remains a common reason for hospitalization and operation. Gastric ulcers may arise from several factors including infection (*Helicobacter pylori*) together with an imbalance between aggressive (acid, pepsin) and protective factors (prostaglandin, mucus and bicarbonate, gastric mucosal blood flow and motility) [1].

The current medicinal treatment of peptic ulcer is generally based on the inhibition of gastric acid secretion by H_2-receptor antagonists (ranitidine, cimetidine) and antimuscarinics, as well as on acid-independent therapy provided by sucralfate and bismuth. In the case of *H. pylori* infection, antibiotics are also used. Drugs providing antisecretory activity coupled with cytoprotective effects could represent a promising approach for the successful treatment of peptic gastric ulcer [2].

In recent years, there has been growing interest in alternative therapies and the use of natural products, especially those derived from plants. In traditional medicine, several plants and herbs have been used to treat gastrointestinal disorders [3].

In this regard, *Chresta martii* (DC.) H. Rob. (Asteraceae), found in the Xingó region (semi-arid area) in Northeastern Brazil, whose genus is considered a synonym for *Argyrovernonia* [4], is recognized by the local population as a traditional herb used to treat gastric diseases [5–7]. This plant has recently been studied botanically and considered as a new species [8] and this is the first report of its chemical profiling and pharmacological properties. The acute toxicity profile of hydroalcoholic extract (HAE) from *C. martii*, the evaluation of its antiulcerogenic effect, as well as its phytochemical analysis with the isolation of a new active natural product is reported here.

Materials and methods

Animals

Male Swiss mice (25–30 g) were housed at 22 ± 2 °C under a 12/12-h light/dark cycle, and food and water were supplied ad libitum. Animals were fasted for 18–24 h before the start of experiments. All efforts were made to minimize animal suffering and the number of animals used. All animal treatments and surgical procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" from the Brazilian Science Society in Laboratory Animals (SBCAL). All experiments were in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by Committee on Animal Ethics (EAEC), Federal University of Pernambuco (process # 23076009313/2003-04).

Plant material and extract preparation

Aerial parts (leaves and flowers) of *C. martii* were collected on 31 July in the Xingó region, Sergipe, Brazil (longitude −37.940 and latitude ranging from −9.5563 to −9.5548, altitude 130 m). After its authentication by Dr. Nádia Roque (Botany Department of Biology Institute—Federal University of Bahia, Brazil) a voucher specimen (protocol # 14602) was deposited in Vale do Acaraú State University herbarium (Sobral, Ceará, Brazil).

Air-dried and powdered aerial parts of the plant (100 g) were extracted using 50 % ethanol at room temperature (28 ± 3 °C) for 48 h. After filtration, the dark green solution was concentrated at 50 °C under reduced pressure to dryness and kept in a freezer. Fresh dilution of dried extract in saline solution (0.9 % NaCl) was prepared on the day of experiments, and administered orally or intraperitoneally in different doses.

Acute toxicity study

The intraperitoneal (i.p.) or enteral (p.o.) acute toxicity LD_{50} of *C. martii* HAE was evaluated in mice ($n = 10$/group) as described previously [9]. Ranging doses (10–2000 mg/kg, i.p. or 50–3000 mg/kg, p.o.) or saline solution (5 mL/kg p.o.) were administered to the animals as a single dose. Animals were observed over a 48-h period for toxicity signs and mortality.

Sub-chronic toxicity study

Body mass loss, liver weight alteration, blood cell count alterations and the biochemical parameters aspartate aminotransferase (AST), alanine aminotransferase (ALT), amylase and lipase were evaluated after once-daily subchronic treatment of HAE (400 mg/kg, p.o.) or saline solution (5 mL/kg p.o.) for fourteen consecutive days. On the 15th day, all the animals were anesthetized with tribromoethanol (200 mg/kg, i.p.), and blood samples were collected from the retro-orbital plexus. Hematology analysis was performed using an automated hematology

analyzer (Pentra 80, Horiba ABX, Montpellier, France). Blood samples were analyzed to measure the following parameters: erythrocyte count, hemoglobin concentration, hematocrit, platelet count, leukocyte count, and differential cell count (neutrophils, monocytes and lymphocytes). For serum biochemistry analysis, the blood was centrifuged at 3000*g* for 15 min after collection. The serum samples were stored at −80 °C prior to analysis. The following serum biochemistry parameters were determined by enzymatic and colorimetric tests from Labtest Diagnóstica (Lagoa Santa/MG, Brazil): AST, ALT, amylase and lipase. After killing, the liver was removed and weighed. Spleen, kidney and heart were macroscopically analyzed for possible ulcerative lesions or hemorrhaging.

Indomethacin-induced gastric lesions

Gastric ulceration was induced in 24-h fasted mice by the administration of indomethacin [40 mg/kg, subcutaneously (s.c.)]. Mice (6 per group) were pre-treated with HAE (100 or 400 mg/kg, p.o.), omeprazole (30 mg/kg, p.o.), used as a positive control, or saline (5 mL/kg; p.o.) 1 h before the challenge. A non-treated group (no indomethacin) was used as control for histological analyses. The animals were killed in a CO_2 chamber 6 h after the ulcerogenic procedure. The stomach was removed and opened along its greater curvature. The ulcer index was evaluated using the quantitative method for assessing the extent of experimental gastric erosions and ulcers as described by Szabo et al. [10]. The percentage inhibition was calculated in relation to the saline group according to the following formula: %inhibition = UIt/UIs × 100, where UIt and UIs correspond to ulcer index of treated and ulcer index of saline groups, respectively.

Histopathological analysis

The H&E-stained gastric samples were semi-quantitatively evaluated for inflammatory infiltration to provide a (0–3) score grade: 0, no infiltration; 1, very mild infiltration; 2, mild infiltration; 3, moderate infiltration; and 4, marked infiltration. These observations were made by an experienced pathologist who was blinded to the treatment protocol.

Statistical analysis

All values are expressed as mean ± SEM. For macroscopic (ulcer index) and histological assessment, the Kruskal–Wallis nonparametric test was used, followed by Dunn's test for multiple comparisons. Student's *t*-test was used to evaluate sub-chronic toxicity data. $P < 0.05$ was considered statistically significant.

HPLC/UV/APCI-MS analysis

The HPLC/UV/APCI-MS analysis of *C. martii* HAE was performed on an HP-1100 liquid chromatography system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a binary pump, a DAD and an autosampler. MS analyses were made on a Finnigan MAT (San Jose, CA, USA) LCQ ion trap mass with an atmospheric pressure chemical ionization (APCI) interface, with the following conditions: capillary temperature, 150 °C; vaporizer temperature, 370 °C; positive mode; sheath gas flow, 60 psi (414 kPa); corona needle current, 5 μA; collision energy, 15 eV. The separation was achieved on a Waters Nova-Pak C_{18} column (250 × 4.6 mm i.d.; 5 μm) eluted with a linear gradient of methanol–water containing 0.1 % formic acid from 10:90 to 100:0 in 40 min. The flow rate was 1 mL/min; UV spectra were recorded at 210, 254 and 366 nm.

Centrifugal partition chromatography

Fractionation was conducted on a counter-current chromatograph CCC-1000 (Pharma-Tech Research Corporation, Baltimore, MD, USA) equipped with dynamic coils of 650 mL total volume. The rotation speed was set at 1000 rpm. Two LC-300 pumps (Scientific Systems Inc., State College, PA, USA) were used to pump either upper or lower phase into the coils at a flow rate of 3.0 mL/min for each phase. The HAE was diluted in 30 mL of a mixture of upper and lower phases (1:1) and introduced through the injection loop. Crude extract (9 g) was chromatographed using the solvent system chloroform–methanol–water (45:11:44) with the upper phase as the mobile phase, followed by phase inversion after 6 h. By this method, the extract was first fractionated into seven major fractions (AI–AVII).

Column liquid chromatography

Fraction AII (2 g) was chromatographed on a silica gel 60 (63–200 μm, Merck, Germany) column (750 × 20 mm). The mobile phase consisted of hexane, ethyl acetate and methanol in increasing polarity proportions. From the 24 fractions obtained, compound **1** (50 mg) could be isolated (Fig. 3).

NMR analyses

^{1}H and ^{13}C NMR spectra were recorded on a Unity Inova 500 spectrometer at 500 and 125 MHz, respectively, with the compound dissolved in CD_2Cl_2. Chemical shifts were recorded in ppm with δ relative to tetramethylsilane (TMS) as internal standard.

Compound **1**

^{1}H-NMR (500 MHz), CD_2Cl_2: δ 1.36 (3 H, s, H-15), 1.38 (3 H, s, H-14), 2.04 (1H, ddd, H-1), 2.51 (1H, m, H-1), 2.54 (H, d, H-2), 2.62 (H, dd, H-2), 2.73 (1H, ddd, H-5), 2.83 (1 H, dd, H-5), 4.51 (2H, d, H-13), 5.43 (1 H, d, H-6), 5.83 (1 H, s, H-9).

^{13}C-NMR (125 MHz), CD_2Cl_2: δ 26.7 (C-14), 27.2 (C-15), 33.9 (C-2), 38.7 (C-1), 47.5 (C-5), 56.0 (C-13), 67.2 (C-6), 74.7 (C-10), 76.2 (C-4), 113.6 (C-9), 121.6 (C-11), 145.1 (C-8), 146.7 (C-7), 168.5 (C-12), 213.1 (C-3).

HRESI/MS 295.1182 $[M+H]^+$, calculated for $C_{15}H_{19}O_6$.

In-vitro inhibition of neutrophil chemotaxis

C. martii HAE and compound **1** were investigated for their ability to inhibit neutrophil chemotaxis in vitro. This evaluation was performed using Boyden's method [11] modified by Zigmond and Hirsch [12].

The collection of chemotactic factor, the collection and preparation of neutrophils and the determination of their migration through the filters were conducted as previously described [13].

Readings were taken for 10 fields of two filters for each sample, and the result was expressed as mean ± SD. The chemotaxis experiment was analyzed using Student's *t* test. $P < 0.05$ was considered statistically significant, and $P < 0.001$ was considered highly significant.

Results

Acute toxicity assay

HAE (50, 100, 2000 or 3000 mg/kg) administered orally (p.o.) did not result in mortality or altered pathognomonic behavior of the central nervous system. Similarly, low doses of HAE (10 or 100 mg/kg) injected intraperitoneally (i.p.) did not change animals' behavior. However, HAE (1000 mg/kg, i.p.) caused 2 deaths up to 6 h after the treatment. Furthermore, HAE (2000 mg/kg; i.p.) caused more than 50 % of mortality up to 6 h after the treatment, followed by 2 and 1 deaths over 24 and 36 h, respectively, from the beginning of the assay. All deaths were preceded by reduction of locomotion and eyelid ptosis.

Sub-chronic toxicity essay

HAE (400 mg/kg, p.o.) treatment for 14 days did not alter blood cell counts, hemoglobin, hematocrit, or liver mass. No macroscopic alterations were observed in internal organs. Indicators of pancreatic function (amylase and lipase) were not altered. ALT and AST values were significantly different from the saline group (Table 1). The body mass curve of the HAE-treated group was not significantly different from that of the saline-treated group (Fig. 1).

Effect of HAE on ulcer index

Indomethacin administration (40 mg/kg, s.c.) produced acute hemorrhagic damage. HAE (100 or 400 mg/kg, p.o.) significantly reduced ($P < 0.001$) the gastric lesion score compared to the saline group. Gastric protection by HAE was similar to the effects observed with omeprazole (30 mg/kg, p.o.) (Table 2).

Histopathological analysis

When compared to the non-treated group (no indomethacin) (Fig. 2a), the gastric mucosa of the saline group (saline + indomethacin) showed marked inflammatory cell accumulation together with disruption of the superficial layers (Fig. 2b). HAE (100 mg/kg) attenuated mucosal erosion as shown in Figure 2c. Histological analysis of omeprazole (30 mg/kg, p.o.) and HAE (100 and 400 mg/kg) groups revealed a significant ($P < 0.05$) decrease in inflammatory cell infiltrate [1 (0–1), 1 (0–1) and 1 (0–1)], respectively (Table 3).

Phytochemical analysis

A fingerprint of the HAE obtained by HPLC/UV/MS analysis is presented in Figure 3. Major peaks were detected in the extract and the presence of flavonoids was excluded. Compounds presented similar UV spectra with a maximum at c. 280 nm and a slight shoulder at 220 nm, which is characteristic of sesquiterpene lactones [14]. The mass spectra of some compounds, such as **1**, showed a low base peak at 295.1, corroborating the range of mass for sesquiterpene lactones.

Compound **1**

Compound **1** could be purified and elucidated as a new sesquiterpene lactone based on the analyses of its spectroscopic data. The ^{1}H and ^{13}C NMR data, together with HMBC correlations, are presented in Table 4.

In-vitro inhibition of neutrophil chemotaxis

When investigated for the ability to reduce leukocyte migration in vitro, HAE significantly reduced the migration distance ($P < 0.05$) at 100 µg/mL. At the same concentration, the isolated sesquiterpene lactone (compound **1**) was able to inhibit leukocyte migration with high significance ($P < 0.001$), which was comparable to the positive control indomethacin (Fig. 4).

Table 1 Effects of HAE on blood parameters (AST, ALT, amylase, lipase and cell count) and liver mass after once-daily sub-chronic treatment of HAE or saline solution for fourteen consecutive days

Blood parameter/treatment	Saline (5 mL/kg p.o.)	HAE (400 mg/kg p.o.)
AST (U/L)	80.69 ± 4.33	110.3 ± 7.24*
ALT (U/L)	51.36 ± 5.54	91.79 ± 8.43*
Amylase (U/dL)	733.2 ± 11.48	683.5 ± 17.92
Lipase (U/dL)	276.0 ± 5.86	268.5 ± 13.73
Erythrocyte (10^6/mm^3)	7.676 ± 0.17	7.793 ± 0.17
Hemoglobin (g/dL)	12.84 ± 0.34	13.14 ± 0.30
Hematocrit (%)	38.67 ± 1.11	40.26 ± 1.05
Total leukocytes (cells/mm^3)	2,080 ± 196.0	2,471 ± 259.8
Neutrophils (cells/mm^3)	211.9 ± 45.35	181.3 ± 66.89
Lymphocytes (cells/mm^3)	1,431 ± 86.20	1,974 ± 212.1
Monocytes (cells/mm^3)	437.3 ± 95.71	316.4 ± 72.83
Platelets (mm^3)	601,300 ± 43,376	514,857 ± 56,340
Liver mass (g)	2.120 ± 0.067	1.925 ± 0.069

Data are presented as mean ± SEM. Student's *t* test

HAE Chresta martii hydroalcoholic extract

* $P < 0.05$ versus saline group

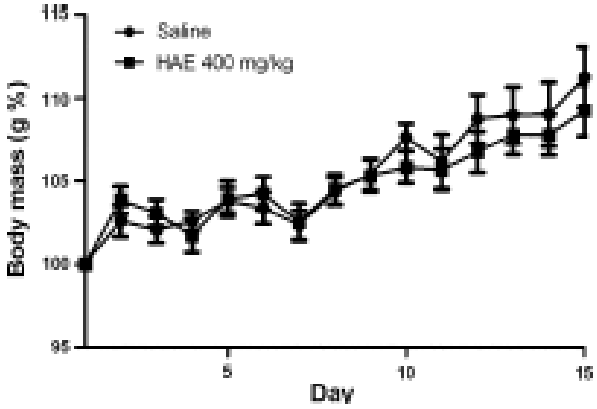

Fig. 1 Body mass evolution of mice treated with HAE (400 mg/kg) for 14 days. Results are expressed as percentage of initial mass mean. There were no significant differences between saline and HAE-treated groups during the experiment

Table 2 Effects of HAE on the ulcer index (macroscopic analysis) inhibition after indomethacin challenge

Treatments	Ulcer index	% Inhibition
Saline	22.25 ± 1.8	–
Omeprazole 30 mg/kg, p.o.	9.31 ± 2.0	58.15*
HAE 100 mg/kg, p.o.	10.09 ± 1.6	54.66*
HAE 400 mg/kg, p.o.	4.16 ± 1.2	81.30*

Kruskal–Wallis nonparametric test followed by Dunn's test was used for multiple comparisons of macroscopic assessment

HAE Chresta martii hydroalcoholic extract

* $P < 0.01$ versus saline group

Discussion

In the acute toxicity study, all the animals treated orally remained alive and did not manifest any significant visible signs of toxicity at the doses evaluated. There were no abnormal signs, behavioral changes, or macroscopic alterations at any time during the observation period. However, HAE at the highest doses (1000 and 2000 mg/kg) given intraperitoneally caused death preceded by observable adverse symptoms. These doses are, respectively, 10 and 20 times the minimum dose which gave gastric protection (100 mg/kg p.o.) in the experimental ulcer model used. This suggests that the extract has a wide safety window. The absence of any deleterious symptoms with oral use denotes low toxicity for this preparation when administrated in single doses.

Serum ALT and AST activities are used as indicators of chemically induced liver damage [15]. The ALT and AST values of our control (saline) group were similar to other reports [16, 17]. Results of the sub-chronic study revealed that there were significant ($P < 0.05$) increases in the means of serum ALT and AST levels in the HAE group when compared to the saline group. The literature shows that 2- to 3-fold increases in ALT levels above the upper limit of normal are needed to be considered indicative of hepatocellular injury [18]. Our study results did not reach these values (1.78 and 1.36 times the saline values of ALT and AST, respectively), and we compared it to mean, not to upper limits. Moreover, these parameters cannot be evaluated in isolation. The determination of this kind of drug-induced toxic effect must be based on the magnitude of the changes in all concurrently evaluated parameters [18]. Other parameters evaluated in our sub-chronic essay (macroscopic organ evaluation, serum lipase and amylase, body mass changes in time, blood parameters and liver mass) did not shown any alterations. Furthermore, the dose used in sub-chronic toxicity was 4 times the therapeutic dose used in this report (400 vs. 100 mg/kg). Pancreatic function indicators, amylase and lipase, did not differ between the HAE and saline groups. These considerations indicate a suitable safety margin for the oral administration of HAE.

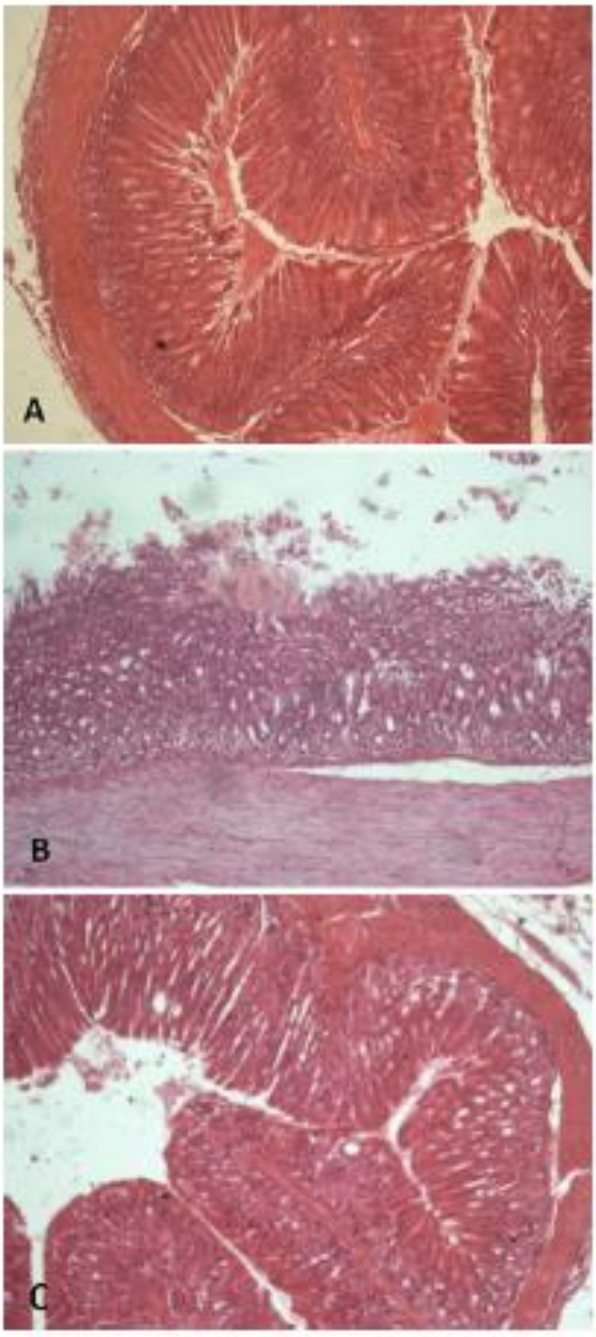

Fig. 2 Photomicrographs of gastric mucosa: a non-treated group with normal conformation of mucosa; b saline group (animals that received indomethacin + saline) showing loss of papillae conformation, with epithelial disruption and gastric lumen enhancement; c animals treated with indomethacin + HAE 100 mg/kg, showing almost totally anatomical conformation, preservation of papillae and gastric luminal space (H&E ×10)

Table 3 Effects of HAE on gastric histological assessment after indomethacin challenge

Treatments	Inflammatory cells score (0–4)
Not treated*	0
Saline	2 (1–3)
Omeprazole 30 mg/kg	1 (0–1)**
HAE 100 mg/kg	1 (0–1)**
HAE 400 mg/kg	1 (0–1)**

Data shown are medians with minimum and maximum scores shown in brackets. Kruskal–Wallis nonparametric test followed by Dunn's test was used for multiple comparisons of histological assessment

HAE, *Chresta martii* hydroalcoholic extract

** $P < 0.05$ versus saline group

* Non-treated group received no indomethacin

Our results clearly demonstrated that HAE protects against indomethacin-induced gastric mucosal lesions in mice, as shown by a significant decrease in the mean scores in comparison to the saline group. The integrity of gastric mucosal defense depends on continuous generation of prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2), mediated by cyclooxygenases 1 (COX1) and 2 (COX2), which catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandins (PGs). PGE_2 and PGI_2 are both potent vasodilators and control many aspects of gastric mucosal defense and healing [19, 20]. These prostaglandins play an important role in maintenance of gastric mucosa blood flow, stimulation of mucus secretion, inhibition of neutrophil adherence and activation, and in the ability to protect the stomach against ulcerogenic agents [21–24]. Leukotrienes, having the same precursor as PGs (arachidonic acid), play an important role in gastric mucosa protective functions. Leukotriene production is related to vasoconstriction and neutrophil chemoattraction [25]. Inhibition of COX causes an imbalance in mucosal levels of leukotrienes and prostaglandins, favoring the production of the former [14].

According to Souza et al. [26], indomethacin-induced gastric lesions are dependent on neutrophil infiltration and nitric oxide (NO) generation through the inducible nitric oxide pathway. Our results of the histological assessment reveal that HAE (100 or 400 mg/kg, p.o.) reduced inflammatory cell migration to the gastric mucosa (Table 3). This reduction in neutrophils may, perhaps, contribute to the gastroprotection exhibited by the HAE.

Previous studies [27] showed that reactive oxygen species (ROS) play a vital role in indomethacin-induced gastric damage via oxidation of important cellular biomolecules such as lipids, proteins and DNA. The ROS-mediated degradation of the cell membrane results in the formation of lipid peroxides and initiates a variety of

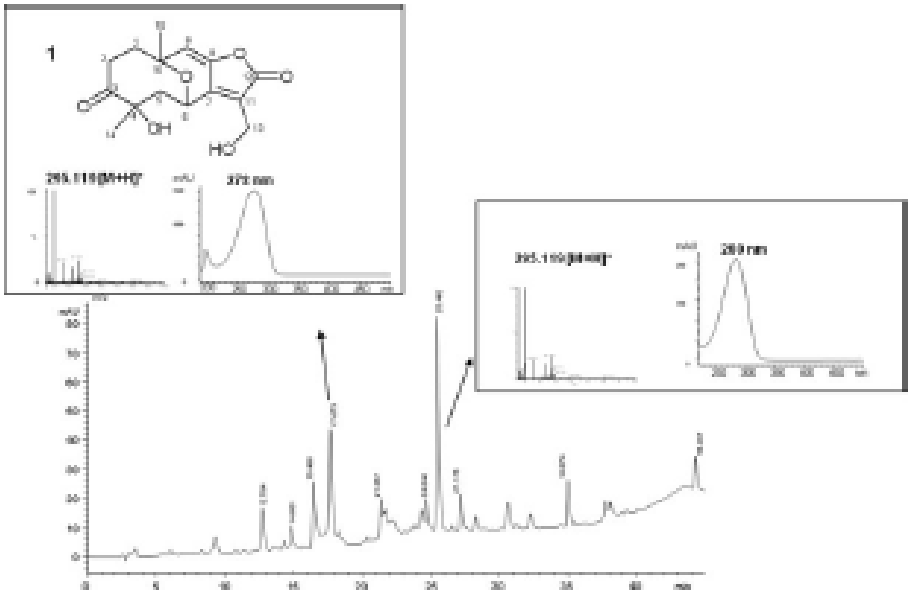

Fig. 3 HPLC/UV/APCI-MS chromatogram of *C. martii* hydroalcoholic extract (C-18 column, UV 254 nm, APCI positive mode, mobile phase: MeOH–H_2O + formic acid 0.2 % in gradient mode) and elucidated structure for compound 1

Table 4 NMR data of compound 1 in CD_2Cl_2

Position	^{13}C NMR	^{1}H NMR	HMBC (^{1}H → ^{13}C)
1	38.7	2.04–2.51	C-2, C-3, C-10, C-15
2	33.9	2.54–2.62	C-3, C-4, C-10
3	213.1		
4	76.2		
5	47.5	2.73–2.83	C-3, C-4
6	67.2	5.43	C-5, C-7, C-8, C-10
7	146.7		
8	145.1		
9	113.6	5.83	C-7, C-8, C-10
10	74.7		
11	121.6		
12	168.5		
13	56.0	4.51	C-7, C-11, C-12
14	26.7	1.38	C-3, C-4, C-5
15	27.2	1.36	C-1, C-9, C-10

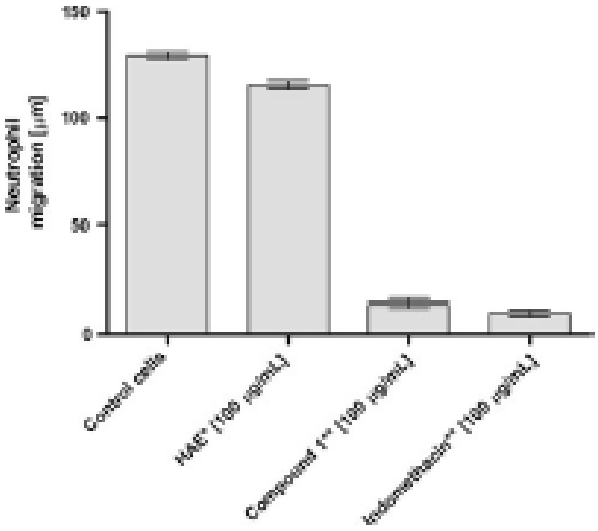

Fig. 4 Effect of HAE and compound 1 on in-vitro neutrophil migration towards a chemotactic factor (lipopolysaccharide) gradient in Boyden chamber chemotaxis assay. Positive control: indomethacin; *$P < 0.05$; **$P < 0.001$

deleterious events, including mucosal lesions, increased vascular permeability and depletion of the mucus layer [28]. Nonprotein sulfhydryl groups, such as glutathione, promote cytoprotection by preventing free-radical oxidative damage in various tissues, including the gastric mucosa [29]. Indomethacin causes gastric erosions with increased lipid peroxidation and decreased glutathione peroxidase activity [27].

Some species from Asteraceae genera (*Eremanthus erythropappus*, *Baccharis illinita* and *Baccharis trimera*, *Senecio brasiliensis*) have demonstrated anti-ulcerogenic activity without deleterious side effects [3, 30–32]. Active compounds of various plants, such as flavonoids, triterpenes and tannins, may be regarded as possible active substances against gastric lesions [33]. In our study, we demonstrated the presence of sesquiterpene lactones in *C. martii* HAE. Some authors have demonstrated that sesquiterpene lactones have gastroprotective effects in experimental models of ulcer [34–39]. In fact, sesquiterpene lactones are related to preservation of endogenous non-protein sulfhydryl groups such as glutathione [40]. These compounds can be related to the gastroprotective

effect observed in our study. The isolated sesquiterpene lactone 1 was able to significantly inhibit neutrophil migration in a Boyden chamber in-vitro assay. This result corroborates the hypothesis that sesquiterpene lactones of this kind are responsible for the observed gastroprotection of HAE.

In conclusion, the low toxicity and the gastroprotective effect of *C. martii* HAE were demonstrated in mice, supporting the traditional use of this plant to treat gastric disorders. This plant provided a new sesquiterpene lactone with neutrophil antichemotactic activity. According to the HPLC/UV/MS fingerprint of *C. martii* HAE, this new plant species seems to be promising in providing new sesquiterpene lactones to be investigated individually for their complete structure elucidation and mechanisms of action in gastric protection.

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Apêndice – B

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Protective effect of *Chresta martii* extract on ethanol-induced gastropathy depends on alpha-2 adrenoceptors pathways but not on nitric oxide, prostaglandins or opioids

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ABSTRACT

Ethnopharmacological relevance: Species of Chresta genus- are recognized by the population of north-eastern Brazil as traditional herbs used to treat gastric diseases and other disorders.
Aim of the study: This work aimed to find out the action mechanism of *Chresta martii* hydro alcoholic extract gastro protective effect in the model of ethanol-induced gastropathy.
Material and methods: Gastropathy was assessed by percentual damaged area determination in photographs of mice opened stomachs. Fasted mice treated with ethanol 99.9% (0.2 ml/animal, p.o.) were pre-treated with *Chresta martii* hydro alcoholic extract (HAE) (50, 100 or 200 mg/kg, p.o.), ranitidine (80 mg/kg, p.o.) or saline (5 ml/kg; p.o.) in different experimental sets, in which pharmacological tools (naloxone, indomethacin, N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) or yohimbine) were added in order to clarify a possible action mechanism. Animals were sacrificed 30 min after ethanol challenge to stomach analysis. Determination of non-protein sulfhydryl groups and tissue hemoglobin, besides histological assessment (H&E) were taken to fully characterize the HAE gastro protective effect.
Results: HAE (100 and 200 mg/kg) was able to protect mucosa against ethanol gastropathy in presence of three (naloxone, indomethacin and L-NAME) of four antagonist/inhibitor tools. The HAE effect was reversed only by yohimbine, showing the alpha-2 adrenoceptors participation on gastro protective effect of this extract. HAE histological characteristics, NP-SH and Hb were compatible with the protective effects.
Conclusions: HAE possesses gastroprotective effects in an ethanol-induced gastropathy model in mice, corroborating the traditional use of this family of plants to treat gastric disorders. This activity is mediated by alpha-2 adrenoceptors activation, but not by nitric oxide release, opioid receptor activation or prostaglandin synthesis. HAE also has antioxidant activity that is thought to either play a role in this biological activity or to be a by-product of alpha-2 adrenergic complex activation.

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1. Introduction

Medicinal floras from caatinga vegetation, a semi-arid region of Brazil, are mainly herbaceous species that are pharmacologically unexplored (Albuquerque et al., 2007). The Asteraceae family represents many species with antiulcer activity, including *Achillea millefolium* L. (Baggio et al., 2003), *Mikania laevigata* (Bighetti et al., 2005), *Fronseria artemisioides*, *Baccharis genistelloides*, *Baccharis rubricaulis*, and *Baccharis illinita* (Gonzales et al., 2000; Verdi et al., 2005).

In this regard, species of the Chresta (Argyrovernonia) genus (Robinson, 1980) are recognized by local populations as traditional herbs used to treat gastric diseases and disorders. According to information from the local community, the aerial part of *C. martii* is prepared as a decoction (~10 g) and consumed to treat gastrointestinal disturbances. However, a literature survey indicates no previous pharmacological validation of this plant (Agra et al., 2008, 2005, 2006). Our recent study (Silva et al., in press) is the first report showing antiulcer effects in other gastropathy models. In this paper, gastroprotective effects were observed without toxicity.

Gastric ulcers may arise from damaging factors, including infection (*Helicobacter pylori*) and imbalances in acid or pepsin, and protective factors, including prostaglandin, mucus and bicarbonate, gastric mucosal blood flow and motility (Sostres et al., 2010). Mucosal defense mechanisms against exogenous factors can be divided into local and neurohormonal mechanisms. As local mechanisms, the vasodilators nitric oxide (NO) and prostacyclin (PGI_2) released by epithelial cells protect mucosa by inhibiting vasoconstrictors (e.g., leukotriene C4, thromboxane A2 and endothelin) and preventing platelet and leukocyte adhesion to vascular walls (Tulassay and Herszényi, 2010). NO also inhibits acid secretion by parietal cells (Berg et al., 2005). Prostaglandins can inhibit gastric acid secretion and stimulate mucus and bicarbonate secretion in the stomach (Wallace, 2008). Neurohormonal mechanisms include gastrin, cholecystokinin, opioids, sympathetic and parasympathetic neurotransmitters.

The central nervous system regulates mucus secretion through the vagus nerve, and central opioid receptor activation enhances mucosa defense (Tulassay and Herszényi, 2010). In addition, peripheral and possibly central alpha-2 adrenoceptor stimulation can mediate anti-secretory action with gastric protection effects (Gyires et al., 2000). This study therefore attempts to elucidate the mechanism of action underlying the gastroprotective effect of *Chresta martii* (DC.) H. Rob. (Asteraceae) on an ethanol-induced gastropathy model.

2. Materials and methods

2.1. Plant material

Specimens of *Chresta martii* (DC.) H. Rob. were collected from the Xingó region in Sergipe, Brazil (longitude 37.940 and latitude ranging from −9.5563 to −9.5548, altitude 130 m) in August 2010. After its identification by Dr. Nádia Roque (Botany Department of the Biology Institute—Federal University of Bahia, Brazil) a voucher specimen was deposited in the Herbarium HUVA (Sobral, Ceará, Brazil) with the number 14602.

2.2. Extract preparation

Air-dried and powdered aerial parts (leaves and flowers) of the plant (100 g) were extracted using 50% ethanol (1 g/100 mL) at room temperature (28 ± 3 °C) for 48 h. After filtration, the dark green solution was concentrated at 50 °C under reduced pressure to dryness and stored in a freezer. A fresh dilution of dried extract in saline solution (0.9% NaCl) was prepared on the day of experiments, and administered by gavage (p.o.) at different doses. The extraction yield was 7%.

2.3. Animals

Male Swiss mice (35–40 g) were housed using standard conditions (22 ± 2 °C under a 12/12 h light/dark cycle; food and water were ad libitum). Animals were fasted for 24 h before the start of experiments. All efforts were made to minimize animal suffering and the number of animals used. All animal treatments and surgical procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" from the Brazilian Science Society for Laboratory Animals (SBCAL). All experiments were approved by the Committee on Animal Ethics (EAEC), Federal University of Pernambuco, Brazil (process no. 23076009313/2003–04).

2.4. Drugs and chemicals

Ranitidine was purchased from Aché Laboratórios Farmacêuticos S.A. (Guarulhos, SP, Brazil). Morphine and naloxone were obtained from Cristália Produtos Químicos Farmacêuticos LTDA (Itapira, SP, Brazil). Clonidine was purchased from Boehringer Ingelheim do Brasil Química e Farmacêutica Ltda (Itapecerica da Serra, SP, Brazil). Yohimbine was purchased from Apsen Farmacêutica (Santo Amaro, SP, Brazil), while 5,5′-dithiobis-(2-nitro-benzoic acid) (DTNB), indomethacin, N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) and L-arginine methyl ester dihydrochloride (L-Arg) were obtained from Sigma Aldrich (St. Louis, MO, USA). Ethanol, formaldehyde, ethylenediaminetetraacetic acid (EDTA), and trichloroacetic acid were purchased from Vetec Química Fina Ltda (Duque de Caxias, RJ, Brazil). Misoprostol was acquired from Biolab Searle (Independência, SP, Brazil), and N-acetyl-cysteine was purchased from Zambon Laboratórios Farmacêuticos (São Paulo, SP, Brazil). All of the drugs, except N-acetyl-cysteine, were solubilized in 0.9% sterile NaCl (saline). The enzymatic kits used for evaluation of tissue hemoglobin were purchased from Labtest Diagnosis (Lagoa Santa, MG, Brazil). All other chemicals were of analytical grade.

2.5. Pharmacological trials

2.5.1. Ethanol-induced gastropathy

Gastric damage was induced in 24 h fasted mice by the administration of ethanol 99.9% (0.2 ml/animal, p.o.). Mice (6/group) were pre-treated with *Chresta martii* hydro alcoholic extract (HAE) (50, 100 or 200 mg/kg, p.o.), ranitidine (80 mg/kg, p.o.) or saline (5 ml/kg p.o.) 1 h before ethanol challenge. An additional group that was not challenged consisted of mice treated with saline only. The animals were euthanatized in a CO_2 chamber 30 min after the damaging procedure. The stomachs were removed and opened along the greater curvature, washed with saline, fixed in glass plates and photographed (Sony Cyber-shot Dsc-h2) at 72 dpi resolution (2816 × 2112 pixels). Hemorrhagic or ulcerative lesions were measured and compared to the total area of each stomach using a planimetry program (ImageJ; National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland, USA).

2.5.2. Histopathological studies

For histological assessment, the glandular stomach was fixed in 10% neutral buffered formalin solution for 24 h, sectioned, and then embedded in paraffin. Sections 4 μm thick were deparaffinized, stained with hematoxylin–eosin (H&E), and examined under a light microscope. The specimens were then assessed according to the criteria of Laine (1988). Briefly, a 1 cm segment of each histological section was assessed for epithelial cell loss (score: 0–3), edema in the upper mucosa (score: 0–4), hemorrhagic damage (score: 0–4), and the presence of inflammatory cells (score: 0–3).

2.5.3. Tissue hemoglobin determination

A standard kit containing Drabkin's reagent for hemoglobin (Hb) determination (LabTest) was used. Portions of fresh gastric tissue (50–100 mg) were homogenized in Drabkin's reagent

solution (100 mg/ml), prepared according to the manufacturer's instructions. The tubes were centrifuged at 10,000 rpm for 10 min. The supernatant was withdrawn, filtrated through 20 μm filters and then centrifuged again. The Hb concentration per 100 mg of tissue was determined by absorbance comparison with a standard Hb dilution at 540 nm.

2.5.4. Non-protein sulfhydryl group (NP-SH) assay

NP-SH, such as glutathione, promotes cytoprotection by preventing free radical oxidative damage in various tissues, including the gastric mucosa (Szabo, et al., 1992). To evaluate the antioxidant activity in the presence of HAE, mice (6/group) received ethanol (99.9%) 1 h before HAE (100 mg/kg, p.o.) or saline (5 ml/kg, p.o.), or 30 min before N-acetyl-cysteine (NAC) (300 mg/kg, i.p.) administration. A saline-only-treated group was also included in the assay. The animals were euthanatized in a CO_2 chamber 30 min after the ulcerogenic procedure, and the stomachs were removed. NP-SH levels were measured using a previously described method (Sedlak & Lindsay, 1968). Briefly, 50–100 mg portions of fresh gastric tissue were homogenized in frozen 0.02 M EDTA (1 ml/100 mg tissue). Then, 400 μl of the homogenate was mixed with 320 μl of distilled water and 80 μl of 50% TCA to precipitate the protein. The tubes were next centrifuged at 3,000 × *g* for 15 min at 4 °C. Finally, 400 μl of supernatant was mixed with 800 μl 0.4 M Tris buffer (pH 8.9) and 20 μl of DTNB, and the mixture was shaken for 3 min. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Tissue levels of NP-SH were reported as mg/g tissue.

2.5.5. Probing the mechanism of action

2.5.5.1. Involvement of opioid receptors in HAE gastric protection. Mice (6/group) were treated with morphine (5 mg/kg, s.c.), HAE (100 mg/kg, p.o.), or saline (5 ml/kg, p.o.). Ethanol challenge was then performed 1 h after the HAE or saline treatments or 30 min after morphine administration. The participation of opioid receptors was evaluated by naloxone (2 mg/kg, i.p.) administration 15 min before the treatments described above. The stomachs were then analyzed as previously described.

2.5.5.2. Role of prostaglandins (PGs) in HAE gastric protection. Mice (6/group) were treated with HAE (100 mg/kg, p.o.), misoprostol (50 μg/kg, p.o.), or saline (5 ml/kg, p.o.) 1 h before ethanol challenge. Indomethacin (10 mg/kg, p.o.) was administered 2 h before HAE, misoprostol, or saline administration. After 30 min, the animals were euthanatized, and the stomachs were analyzed as described previously.

2.5.5.3. Role of nitric oxide (NO) in HAE gastric protection. Mice (6/group) were treated with HAE (100 mg/kg, p.o.), L-Arg (600 mg/kg, i.p.), or saline (5 ml/kg, p.o.). Ethanol challenge was performed 1 h after HAE or saline treatments or 30 min after L-Arg administration. L-NAME (20 mg/kg, i.p.), a nonspecific inhibitor of nitric oxide synthase (NOS), was administered 15 min before the treatments described above. The animals were euthanatized in a CO_2 chamber 30 min after ethanol challenge, and the stomachs were removed and analyzed according to the methods described previously.

2.5.5.4. Role of alpha-2 adrenoceptors in HAE protection. Alpha-2 adrenoceptors involvement was accessed by the administration of yohimbine (2 mg/kg, s.c.) in mice (6/group) 20 min before HAE (100, 200 or 400 mg/kg, p.o.), clonidine (80 mg/kg, p.o.), or saline (5 ml/kg, p.o.). Ethanol challenge was performed 1 h after these treatments. The animals were euthanatized in a CO_2 chamber 30 min after ethanol challenge and analyzed as described previously.

2.6. Statistical analysis

All values are expressed as the means ± S.E.M. For ulcerative lesion area assessment, an ANOVA test was used followed by Bonferroni's test for multiple comparisons. For histological assessment, the Kruskal–Wallis nonparametric test was used followed by Dunn's test for multiple comparisons. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Protective effect of HAE in ethanol-induced gastropathy

Ethanol administration (0.2 ml/mice) produced acute hemorrhagic striations in the gastric mucosa (Fig. 1). HAE (50 mg/kg) did not protect the gastric mucosa against ethanol damage when compared to the saline-treated group (17.7 ± 3.7 versus 21.6 ± 3.4 injured area %). HAE (100 or 200 mg/kg) significantly reduced ($P < 0.05$) the percent area of gastric lesions compared to the saline treated group (4.9 ± 1.2 and 3.0 ± 1.4 versus 21.6 ± 3.4 injured area %), as did ranitidine (6.3 ± 1.9 versus 21.6 ± 3.4 injured area %).

3.2. Histopathological studies

Table 1 and Fig. 2 demonstrate the capacity of ethanol to induce a high degree of epithelial cell loss and edema as well as hemorrhagic patch formation on the gastric tissue. Such observations were significantly reduced ($P < 0.05$) in animals administered HAE (100 or 200 mg/kg).

3.3. Tissue Hb and NP-SH determination

Table 2 shows that HAE (100 mg/kg) treatment significantly increased ($P < 0.5$) the gastric mucosal NP-SH levels and promoted a marked reduction in Hb levels when compared to saline-treated animals.

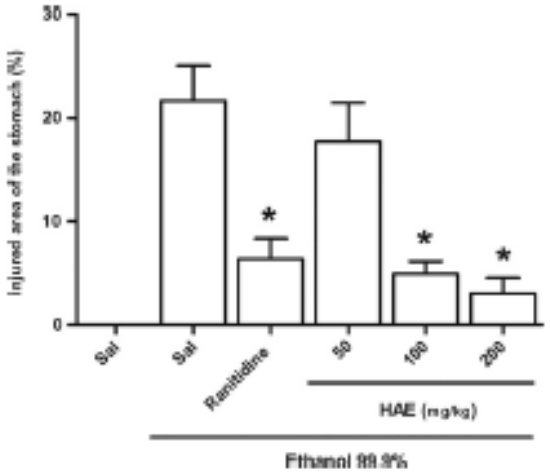

Fig. 1. Effect of HAE on ethanol-induced gastropathy. Mice were treated orally with saline (Sal), ranitidine or HAE. After 60 min, absolute ethanol (0.2 ml per animal) was administered. The control group was treated with saline only. Gastric damage was quantified after 30 min. The results are expressed as the mean ± SEM for each group of six mice. *$P < 0.05$ in relation to the ethanol+saline group. ANOVA followed by Bonferroni's Multiple Comparison Test.

Table 1
Protective effect of *Chresta martii* hydro-alcoholic extract (HAE) on ethanol-induced microscopic damage in the gastric mucosa.

Experimental group (n=6)	Hemorrhagic damage (score 0–4)	Edema (score 0–4)	Epithelial cell loss (score 0–3)	Inflammatory cells (score 0–3)	Total (score 0–14)
Saline	0	0	0	0	0
Ethanol + Saline	2 (0–4)	2 (1–4)	3 (2–4)	0	6 (3–11)
Ethanol + Ranitidine (80 mg/kg)	0 (0–3)	1 (0–2)*	1 (0–2)*	0	2 (0–7)*
Ethanol + HAE (50 mg/kg)	1 (0–4)	2 (1–3)	3 (2–3)	0	5.5 (3–10)
Ethanol + HAE (100 mg/kg)	0 (0–0)*	1 (1–2)	2 (0–2)*	0	2.5 (1–4)*
Ethanol + HAE (200 mg/kg)	0 (0–0)*	0 (0–0)*	1 (0–1)*	0	1 (0–1)*

The table shows median values followed by minimum and maximum scores (in brackets). Kruskal–Wallis nonparametric test followed by Dunn's test.

* $P < 0.05$ compared to the ethanol + saline group.

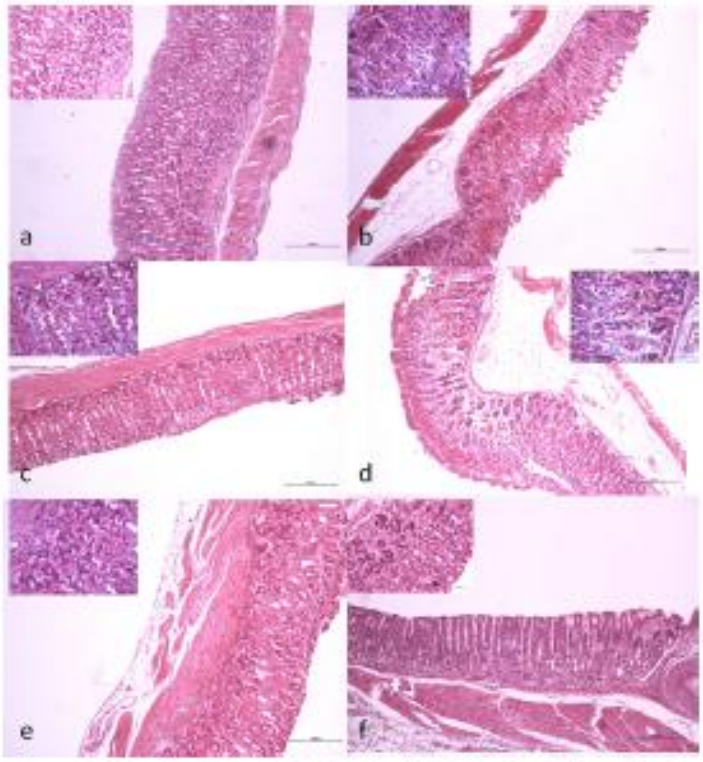

Fig. 2. Photomicrographs of gastric mucosa (100×, and detail in box at 400×). (a) Saline only; (b) Ethanol + saline, showing disruption of the gastric gland superficial region with epithelial cell loss, intense hemorrhage and edema in conjunctive adjacent tissue; (c) Ethanol + ranitidine (80 mg/kg); (d) Ethanol + HAE (50 mg/kg); (e) Ethanol + HAE (100 mg/kg), showing almost complete preservation of the morphologic characteristics of the mucosa; (f) Ethanol + HAE (200 mg/kg), also showing almost complete preservation of the morphologic characteristics of the mucosa.

3.4. *Involvement of opioid receptors in HAE gastric protection*

Morphine (5 mg/kg, s.c.) treatment showed a gastro-protective effect on ethanol-induced gastropathy that was readily reversed by naloxone (2 mg/kg, i.p.). HAE (100 mg/kg) significantly reduced ($P < 0.05$) the ethanol-induced damages with or without prior naloxone injection (4.9 ± 1.2 versus 21.6 ± 3.4 and 4.1 ± 0.9 versus 21.6 ± 3.4 injured area %, respectively) (Fig. 3).

3.5. *Role of prostaglandins in HAE gastric protection*

The protective effect of misoprostol (50 μg/kg, p.o.) in ethanol-induced gastropathy was counteracted by indomethacin (10 mg/kg, p.o.). Ethanol- and ethanol plus indomethacin-induced damages were significantly ($P < 0.05$) reduced by HAE (100 mg/kg) (20.7 ± 3.6 versus 4.9 ± 1.2 and 24.7 ± 2.8 versus 5.2 ± 2.2 injured area %, respectively) (Fig. 4).

3.6. *Involvement of NO in HAE gastric protection*

Ethanol-induced gastropathy was reversed by treatment with ranitidine. Simultaneous administration of ethanol and L-NAME (20 mg/kg, i.p.) produced hemorrhagic damage that could be partially reversed by L-Arg (600 mg/kg, i.p.) injection. In both situations, with or without L-NAME pre-treatment, HAE (100 mg/kg) significantly ($P < 0.05$) reduced the damaged area (4.9 ± 1.2 versus

Table 2
Effect of *Chresta martii* hydro-alcoholic extract (HAE) on tissue concentration of hemoglobin (Hb) and non-protein sulfhydryl group (NP-SH) after ethanol (99.9%) challenge.

Experimental group (n = 6)	Tissue Hb (μg/100 mg tissue) ± S.E.M.	Tissue NP-SH (mg/g) ± S.E.M.
Saline	9.4 ± 0.7	269.3 ± 64.1
Ethanol + Saline	12.5 ± 0.3	113.8 ± 7.0
Ethanol + NAC	–	175.0 ± 17.1
Ethanol + HAE (100 mg/kg)	9.5 ± 0.4*	338.0 ± 67.2*

The table shows means ± SEM. ANOVA followed by Bonferroni's Multiple Comparison test.
* $P < 0.05$ compared to the ethanol + saline group.

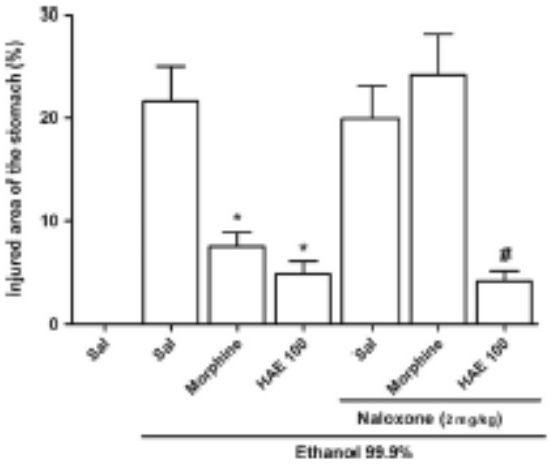

Fig. 3. Lack of involvement of opioid receptors in HAE gastro-protection in an ethanol-induced gastropathy model. The mice were treated orally with saline (Sal), morphine (5 mg/kg) or HAE (100 mg/kg). A second set of mice with naloxone (2 mg/kg) pretreatment was used to evaluate opioid participation in HAE effects. The results are expressed as the mean values ± SEM for each group of six mice. *$P < 0.05$ in relation to the ethanol + saline group. #$P < 0.05$ in relation to the ethanol + naloxone + saline group. ANOVA followed by Bonferroni's Multiple Comparison Test.

21.6 ± 4.1 and 7.5 ± 1.4 versus 21.0 ± 3.2 injured area %, respectively) (Fig. 5).

3.7. Involvement of alpha-2 adrenoceptors in HAE gastric protection

Clonidine (80 mg/kg, p.o.) pre-treatment significantly ($P < 0.05$) protected the mouse stomachs against ethanol challenge (3.5 ± 1.1 versus 18.3 ± 3.4 injured area %). Pre-administration of yohimbine (2 mg/kg, s.c.) reversed the clonidine and HAE (100 or 200 mg/kg) protective effects (17.0 ± 1.0 versus 10.4 ± 1.6, 18.8 ± 1.4 and 12.4 ± 3.5 injured area %, respectively). However, administration of higher doses of HAE (400 mg/kg) restored its protective effect (4.1 ± 0.9 versus 17.0 ± 1.0 injured area %) (Fig. 6).

4. Discussion

We explored the gastroprotective properties of *Chresta martii* (Asteraceae) hydro-alcoholic extract (HAE) in mice. Ethanol ingestion is characterized by the development of gastric mucosal

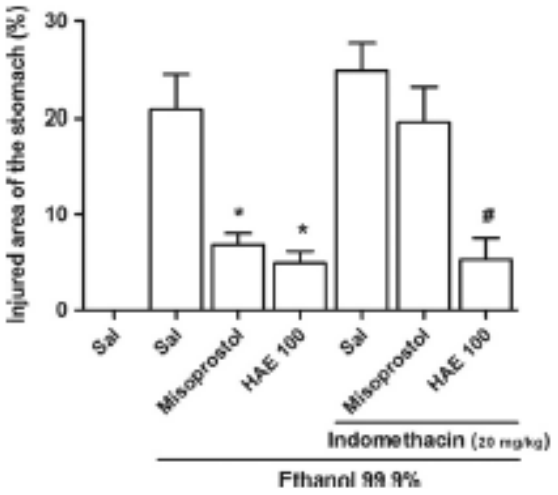

Fig. 4. Lack of involvement of prostaglandins in HAE gastroprotection in an ethanol-induced gastropathy model. The mice were treated orally with saline (Sal), misoprostol (50 μg/kg) or HAE (100 mg/kg). A second set of mice with indomethacin (10 mg/kg) pretreatment was used to evaluate prostaglandin participation in HAE effects. The results are expressed as the mean values ± SEM for each group of six mice. *$P < 0.05$ in relation to the ethanol + saline group. #$P < 0.05$ in relation to the ethanol + indomethacin + saline group. ANOVA followed by Bonferroni's Multiple Comparison Test.

lesions in humans (Laine and Weinstein, 1988). In the model used in this study, absolute ethanol is the main factor that leads to intense damage of the gastric mucosa and induces multiple hemorrhagic red bands (patches) of different sizes along the long axis of the glandular stomach (Mincis et al., 1995). Gastric injury induced by ethanol is linked to oxygen free radical production in quantities that are directly proportional to the ethanol dose. The damage of the gastric cells is closely linked with the intensity of superoxide anion production (Mutoh et al., 1990), which induces intracellular oxidative stress and mitochondrial depolarization, resulting in mucosa cell death (Hirokawa et al., 1998). Other mechanisms of injury include the release of inflammatory mediators, inducing vasoconstriction and ischemia followed by cell death. In addition, gastric blood flow stasis and microvascular disruption lead to hemorrhage and necrotic tissue injury (Szabo et al., 1985).

Our results showed a protective effect for HAE in an ethanol-induced gastropathy model. Hb concentration in mucosal tissue, which is indicative of mucosal bleeding, was diminished in the HAE-treated groups. In addition, HAE also showed protective effects through the maintenance of NP-SH levels in macerated gastric mucosa. The role of endogenous NP-SH in mucosal protection has already been demonstrated in models of ethanol-induced gastric injury (Loguercio et al., 1993). N-acetylcysteine (NAC), the acetylated variant of the amino acid L-cysteine, is a source of sulfhydryl (SH) groups and is converted in the body into metabolites capable of stimulating glutathione (GSH) synthesis. The presence of GSH promotes detoxification, and GSH can act as a free radical scavenger, specifically for reactive oxygen species (Kelly, 1998). Therefore, the effect of HAE on NP-SH levels in the gastric mucosa may be an important element in HAE protective action. The significant reduction observed in edema and hemorrhagic scores as well as the preservation of stomach epithelium in samples from animals treated with HAE are also evidence of its gastroprotective activity.

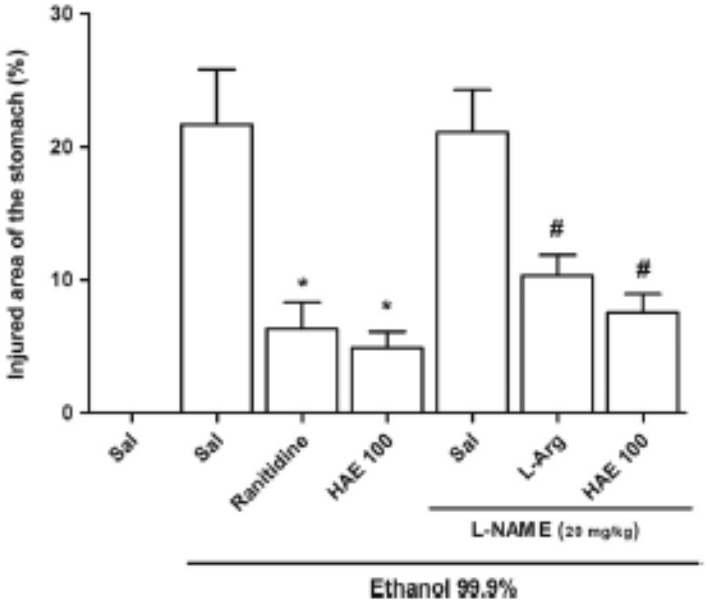

Fig. 5. Lack of involvement of NO in HAE gastroprotection in an ethanol-induced gastropathy model. The mice were treated orally with saline (Sal), L-Arg (600 mg/kg) or HAE (100 mg/kg). A second set with L-NAME (20 mg/kg) pretreatment was used to evaluate the possibility of NO participation in HAE effects. The results are expressed as the mean values ± SEM for each group of six mice. *$P < 0.05$ in relation to the ethanol + saline group. #$P < 0.05$ in relation to the ethanol + L-NAME + saline group. ANOVA followed by Bonferroni's Multiple Comparison Test.

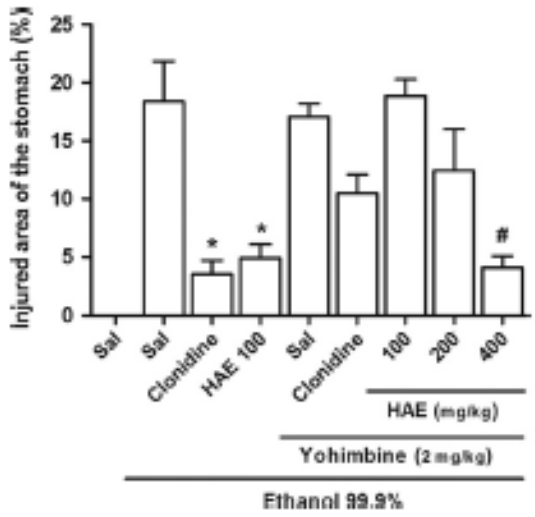

Fig. 6. Participation of alpha-2 adrenoceptors in HAE gastroprotection in an ethanol-induced gastropathy model. The mice were treated orally with saline (Sal), clonidine (0.0 mg/kg) or HAE (100 mg/kg). Yohimbine (02 mg/kg) pretreatment prevented HAE (100 and 200 mg/kg) gastroprotection. Four times the minimal gastroprotective dose of HAE reversed yohimbine impairment. The results are expressed as the mean values ± SEM for each group of six mice. *$P < 0.05$ in relation to the ethanol + saline group. #$P < 0.05$ in relation to the ethanol + yohimbine + saline group. ANOVA followed by Bonferroni's Multiple Comparison Test.

Based on these results, we decided to determine the lowest (100 mg/kg) HAE dose with a significant protective effect to help identify a possible mechanism of action.

Opioids have been found to exert protective effects on ethanol-induced lesions, and both mu and delta opioid receptors were thought to be involved in mucosal protective action (Gyires et al., 2000). In our study, the mucosal protection exerted by morphine was readily antagonized by the non-selective opioid receptor antagonist naloxone. Meanwhile, the protective effect of HAE remained despite pre-treatment with this antagonist.

PGs are a class of mediators produced by cyclooxygenase enzymes 1 and 2 (COX-1 and COX-2). PGs possess important roles in the gastrointestinal tract, stimulating mucus and bicarbonate secretion in the stomach and mediating the maintenance of an optimal pH gradient at the mucosal surface. The beneficial effects of prostaglandin analogs, such as misoprostol (an analog of PGE_1), in humans have been observed at doses that produce significant inhibition of gastric acid secretion (Wallace, 2008; Palileo and Kaunitz, 2011). Our results showed that HAE gastroprotection remained even with the prior administration of indomethacin (a dual inhibitor of COX-1 and COX-2). Furthermore, HAE protection was similar to that observed with misoprostol administration.

NO exhibits many of the same functions in the gastrointestinal tract as prostaglandins, including stimulation of mucus secretion as well as promoting healing and maintenance of the mucosal blood flow (Wallace, 2006; Tulassay and Herszényi, 2010). NO is synthesized by the conversion of L-arginine to equimolar amounts of L-citrulline and NO (Moncada and Higgs, 1993). Our work demonstrated that the gastroprotection of HAE did not directly depend on NO generation, because it was the same regardless of prior administration of the NO synthesis inhibitor L-NAME. Moreover, according to the results, we can assert that the HAE protective activity does not depend upon PGs or the opioid system.

In our study, previous administration of yohimbine, an alpha-2 adrenoceptor antagonist, reversed the gastroprotective effects of low doses of HAE (100 and 200 mg/kg). Quadrupling the dose

of HAE to 400 mg/kg nullified the effect of yohimbine. The competition for adrenergic receptors possibly favored the binding of extract molecules at increasing doses. It is well established that alpha-2 adrenergic receptors mediate various responses in the gastrointestinal tract, such as gastric acid secretion, and effectively inhibit both chemically and physically induced gastric lesions. Therefore, because alpha-2 agonists, such as clonidine, are effective against a variety of mucosal lesions, a complex mechanism of gastroprotection is proposed. In this mechanism, activation of alpha-2 receptors most likely overcomes multiple mechanisms of gastric insult (Gyires et al., 2000).

Both central and peripheral alpha-2 adrenoceptors play an important role in the modulation of gastric acid secretion. While the mechanisms by which central alpha-2 adrenoceptors promote the inhibition of acid output remain poorly understood, at the peripheral level, it was shown that presynaptic alpha-2 adrenoceptors, located on cholinergic terminals of the vagus nerve, modulate gastric secretion through the inhibition of acetylcholine release (Blandizzi et al., 1995). A recent review (Schubert, 2009) showed that acetylcholine, released from postganglionic neurons, directly stimulates the parietal cell via muscarinic-3 (M3) receptors. This process is coupled to the activation of phospholipase C with the generation of inositol triphosphate and an increase in intracellular calcium concentration that leads to acid secretion. Acetylcholine also acts indirectly by activation of M2 and M4 receptors on somatostatin-containing D cells of gastric glands, inhibiting somatostatin secretion and thus removing the tonic restraint exerted by this peptide on parietal cells. Our results may indicate an inhibition of acetylcholine release, among other possibilities, leading to decreased acid secretion upon the interaction of HAE with alpha-2 adrenoceptors. Finally, our study showed that oral administration of the smallest doses of HAE (100 and 200 mg/kg), in an experimental model of ethanol-induced gastropathy had gastroprotective effects in 100% of the animals treated. These results corroborated the traditional use of *C. martii* to treat gastrointestinal disorders and indicated its therapeutic potential when used to repair gastric mucosal lesions.

5. Conclusions

HAE possesses gastroprotective effects in an ethanol-induced gastropathy model in mice, corroborating the traditional use of this family of plants to treat gastric disorders. This activity is mediated by alpha-2 adrenoceptors activation, but not by nitric oxide release, opioid receptor activation or prostaglandin synthesis. HAE also has antioxidant activity that is thought to either play a role in this biological activity or to be a byproduct of alpha-2 adrenergic complex activation.

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