

**ATIVIDADE ANTIULCEROGÊNICA DE  
ALCALOIDES PIRROLIZIDÍNICOS  
OBTIDOS A PARTIR DO  
EXTRATO ETANÓLICO DE *SENECIO  
BRASILIENSIS***

**PERSPECTIVAS DE UMA NOVA TERAPÊUTICA PREVENTIVA  
E CURATIVA DAS ÚLCERAS GÁSTRICAS E DUODENAIIS**

**Walber Toma**



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ETANÓLICO DE *SENECIO BRASILIENSIS*: PERSPECTIVAS DE  
UMA NOVA TERAPÊUTICA PREVENTIVA E CURATIVA DAS  
ÚLCERAS GÁSTRICAS E DUODENAIS**

*Este exemplar corresponde à versão final da Tese de Doutorado,  
apresentada à Pós-Graduação da Faculdade de Ciências Médicas -  
UNICAMP, para obtenção do Título de Doutor em Farmacologia do  
Farmacêutico - Walber Toma.*

*Campinas, 12 de dezembro de 2003.*

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**CAMPINAS**

**2003**

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**WALBER TOMA**

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**2003**

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# **RESUMO**

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*Senecio brasiliensis* é uma planta nativa da América do Sul e comumente encontrada no Brasil. Popularmente é conhecida como Margaridinha, Flor-das-Almas, Tasneirinha e é muito utilizada pela medicina popular para o tratamento de dores estomacais. Estudos fitoquímicos relatam a presença de Alcalóides Pirrolizidínicos (PAs) em *Senecio brasiliensis*. Este estudo tem portanto como objetivo isolar e avaliar a atividade antiulcerogênica dos PAs em modelos tradicionais de indução de úlceras gástricas e duodenais em roedores. Além disso, enfatiza também o(s) provável(eis) mecanismo(s) de ação antiulcerogênico dos PAs. Os resultados obtidos demonstram que os PAs apresentam eficácia antiulcerogênica gástrica e duodenal, bem como demonstra mecanismos farmacológicos que evidenciam uma cicatrização das lesões com caráter não apenas quantitativo, mas também qualitativo, sendo portanto provável protótipo de droga com grandes perspectivas futuras no combate aos males das lesões gástricas e duodenais.

# **1. INTRODUÇÃO**

## 1.1- Úlcera péptica

As úlceras pépticas são lesões crônicas que, na maioria das vezes, aparecem em qualquer porção do trato gastrointestinal exposta à ação agressiva do suco acido-péptico. Estas lesões, geralmente recidivantes, são diagnosticadas mais frequentemente em adultos de meia-idade ou mais velhos, mas que podem tornar-se evidentes, pela primeira vez, no início da vida adulta (CALAM e BARON, 2001).

Dados epidemiológicos relatam que 5 a 10% de todos os indivíduos desenvolvem úlcera péptica ao longo de suas vidas. Embora a doença ulcerosa seja uma causa comum de morbidade, esta raramente leva à morte (BRUNTON, 1996).

A capacidade da mucosa gastrointestinal em resistir à agressão por agentes endógenos e exógenos pode ser atribuída a uma associação de fatores denominados como elementos defensivos da mucosa gastrointestinal (CALAM e BARON, 2001).

Nas últimas décadas, tem sido relatado que as úlceras pépticas, em geral, não dependem só da acidez gástrica, mas também da presença de fatores predisponentes os quais atuam coletivamente reduzindo a defesa da mucosa gástrica contra a ação de diferentes agentes lesivos, outros que não o ácido clorídrico e a pepsina, naturalmente presentes no estômago (Wallace, 2001). Esta relação também foi estudada por PESKAR e MARICIC (1998), o que estabeleceram que as úlceras pépticas seriam resultantes de uma diminuição dos fatores defensivos da mucosa, ou seja, das forças de resistência ao suco gástrico.

Dentre estes fatores estão a secreção de muco e bicarbonato, além das prostaglandinas citoprotetoras.

Atualmente, diversos trabalhos têm relatado também o envolvimento de hormônios gastrointestinais no processo secretório ácido gástrico e, conseqüentemente, na fisiopatologia da úlcera péptica. Dentre estes estão gastrina e somatostatina, os quais auxiliam na modulação da função secretória no trato gastrointestinal (DOCKRAY, 1999).

A gastrina é um hormônio normalmente secretado pelas células G presentes na região do antro gástrico e do intestino delgado. A presença de proteínas presentes numa refeição, a distensão da parede gástrica e o aumento do pH intragástrico são os principais estímulos que promovem a secreção deste hormônio pelas células G. O principal efeito fisiológico da gastrina é a estimulação da secreção ácida gástrica pelas células parietais na

mucosa oxíntica do estômago (DOCRKAY, 1999). Este aumento de secreção, mediado pela gastrina, pode ser proporcionado pela estimulação direta do receptor de gastrina/CCK<sub>B</sub> presente nas células parietais, ou pela promoção de um aumento na secreção de histamina, um agonista de receptores H<sub>2</sub> nas células parietais e que também estimula a secreção de ácido gástrico (CALAM e BARON, 2001)

Uma pequena quantidade de histamina (formada continuamente na mucosa gástrica, quer em resposta ao ácido no estômago ou por outras razões), causa secreção muito pequena de ácido. Entretanto, sempre que acetilcolina ou gastrina estimulam simultaneamente as células parietais, a histamina mesmo em pequenas quantidades, aumenta muito a secreção de ácido (MAKOVEC et al., 1999).

Atuando em receptores H<sub>2</sub>, a histamina é liberada através de um mecanismo parácrino por células enterocromafins-like (ECL), semelhantes aos mastócitos. As células ECL, existentes na lâmina própria do estômago e dispostas em íntimo contato com as células parietais, liberam histamina que promove aumento da secreção ácida por elevar os níveis intracelulares de AMPc (KONTUREK et al., 1995).

A somatostatina é um polipeptídeo secretado pelas células D presentes na região do antro e do fundo do estômago, além do pâncreas, também contribui na proteção da mucosa gástrica inibindo a função secretória ácida das células G de gastrina e ECL. As atividades biológicas da somatostatina, no trato gastrointestinal, incluem a inibição da secreção de ácido clorídrico e de pepsina no estômago (ZAKI et al., 2002).

Em adição, também é consenso que a secreção ácida e sua regulação, não podem ser consideradas como os únicos responsáveis pela úlcera péptica (o que os tornaria alvos farmacológicos restritos na remissão), já que um número significativo de pacientes ulcerados apresenta quantidades normais de ácido no estômago. Este fato levou, nos últimos anos, ao estudo e a descoberta da bactéria *Helicobacter pylori*, presente na mucosa da grande maioria de pacientes com úlceras gástricas e duodenais. Por outro lado, também foi demonstrado que esta bactéria pode estar presente na mucosa de indivíduos sadios que podem ou não desenvolver doença ulcerosa. Assim, a presença da *H. pylori* e as alterações da secreção ácida, são hoje consideradas como agentes importantes na etiologia de úlceras gastrointestinais na espécie humana (BRUNTON, 1996).

Além dos mecanismos secretórios e citoprotetores, tem sido destacada a participação dos radicais livres de oxigênio gerados em processo de oxidação, que na mucosa gastroduodenal causam lesões pépticas. Os radicais livres são moléculas que agredem a membrana lipídica (peroxidação lipídica), ácidos nucleicos, enzimas e receptores, ocasionando alterações na estrutura, além de alterações na atividade celular (Andreoli, 2000). Diversos trabalhos relatam que nas induções de úlceras pépticas em ratos pela técnica de isquemia seguida de reperfusão (isquemia-reperfusão), estresse, etanol e drogas antiinflamatórias não esteroidais (DAINES) ocorre participação destes radicais livres na fisiopatologia das lesões. Além disso, tem sido sugerido que os radicais livres gerados por neutrófilos devem possuir um importante fator na formação destas lesões em úlceras crônicas induzidas pela administração de ácido acético (ITO et al., 1998).

Dentre estes radicais livres destacam-se os derivados do oxigênio, compostos por 4 elétrons, o ânion superóxido ( $O_2^-$ ), o peróxido de hidrogênio ( $H_2O_2$ ) e o radical hidroxila (OH), que são reduzidos a 1, 2 e 3 elétrons, respectivamente. Por outro lado, sabe-se que a isquemia-reperfusão é capaz de promover aumento de radicais livres derivados do oxigênio seguido de peroxidação lipídica trazendo, como consequência, as lesões gástricas (ITO et al., 1998).

LA CASA et al. (2000), relatam que o processo de peroxidação lipídica resulta no aumento da atividade da enzima Xantino-Oxidase (XO) que, por sua vez induz aumento da produção e da liberação de radicais livres. Isto traz como consequência também a ativação dos leucócitos polimorfonucleares (PMN), que migram para o espaço intersticial e com isso promovem a liberação de mais radicais livres, ocasionando consequentemente inflamação e lesão do tecido (MOTILVA et al., 1996). VILLEGAS et al. (2001) relata a importância da enzima mieloperoxidase (MPO), uma vez que esta enzima serve como marcador enzimático de leucócitos e permitindo, portanto, a quantificação da ocorrência do processo inflamatório no tecido lesado.

ARUOMA (1996) demonstra que os níveis de peroxidação lipídica, após a lesão da mucosa gástrica, podem ser avaliados através da quantificação dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBA RS).

No processo oxidativo existem também enzimas que atuam como protetoras do processo de peroxidação lipídica. É o caso da superóxido desmutase (SOD) e da glutathione

peroxidase (GSH-px), por exemplo. Diversos estudos relatam o aumento quantitativo dos níveis destas enzimas em processos de oxidação lipídica (TANAKA e YUDA, 1993).

Vários agentes endógenos, como, por exemplo, os compostos sulfidrílicos (SH), são capazes de reduzir a formação de radicais livres e, conseqüentemente, a lesão celular. Os compostos sulfidrílicos são, portanto, substâncias gastroprotetoras capazes de manter um alto fluxo sanguíneo na mucosa gástrica, o que reduz a lesão tecidual (ARUOMA, 1996)

Nos últimos anos, os estudos científicos da fisiopatologia das úlceras gástricas avaliam a participação de outros elementos importantes no controle da manutenção da homeostase do trato gastrointestinal. Componentes que poderiam auxiliar não apenas no processo de cicatrização das lesões gastroduodenais, como também contribuir na qualidade da cicatrização destas lesões, evitando assim a rescidiva das mesmas, tem sido investigados (KONTUREK et al., 2001). Dentre estes elementos encontra-se o fator de crescimento epidermal (EGF). O EGF é um polipeptídeo de 53 aminoácidos secretado principalmente pelas glândulas salivares, pâncreas, fígado e glândulas mamárias. Grandes quantidades de EGF também são secretadas por células presentes em todo o trato gastrointestinal (ELLIOT et al., 2000). Depois de secretado, o EGF acopla-se a receptores celulares de EGF o que, por sua vez, estimula assim importantes elementos envolvidos no processo de cicatrização celular tais como angiogênese e re-epitelização do tecido lesado (SZABO e VINCZE, 2000).

Na mucosa gastroduodenal, o EGF está envolvido, portanto, na regulação do crescimento e na re-epitelização da mucosa gastrointestinal sob condições fisiológicas normais, e com isso, o EGF exerce uma ação gastroprotetora (TARNAWSKI et al., 1998).

Apesar do amplo conhecimento da fisiopatologia das úlceras pépticas no trato gastrointestinal, da diversidade de drogas utilizadas no tratamento desta patologia e do avanço científico-tecnológico das indústrias farmacêuticas na busca de novos medicamentos, não há ainda uma droga que produza 100% de remissão nas úlceras gastroduodenais (WALLACE, 2001). Deste modo, a busca de novos fármacos que reduzam ao máximo esta rescidiva torna-se um importante alvo de pesquisa para a indústria farmacêutica; as plantas medicinais ganham, dia após dia, um grande papel nesta linha de pesquisa, já que se constituem em “laboratórios”, de síntese de moléculas, extremamente importantes e inovadores.

Nos últimos anos, a Organização Mundial de Saúde (OMS) estimou que 80% dos países em desenvolvimento utilizam-se da medicina tradicional; destes, 85% fazem uso das plantas medicinais ou seus derivados como fonte de substâncias potencialmente ativas em diversas patologias, incluindo os distúrbios gastrointestinais (SHELDON et al., 1997). As plantas medicinais são, portanto, fonte importante de moléculas biologicamente ativas, muitas dos quais acabam por servir de modelo para a síntese de um grande número de fármacos (WALL et al., 1988).

No Brasil, estima-se que 25% dos US\$ 8 bilhões de faturamento, em 1996, da indústria farmacêutica nacional sejam originados de medicamentos derivados de plantas. Além disso, apenas 20% da população em nosso país é responsável por 63% do consumo de medicamentos disponíveis. O restante da população utiliza produtos de origem natural, especialmente plantas medicinais, como única fonte de recursos terapêuticos (GARCIA et al., 1996).

O Brasil é o país com a maior diversidade genética vegetal do mundo, contando com mais de 55.000 espécies catalogadas (DIAS, 1996) de um total estimado entre 350.000 a 550.000. Dentre estas espécies, encontra-se *Senecio brasiliensis* (Figura 1), uma planta nativa da América do Sul e pertencente à família Asteraceae. Popularmente, *S. brasiliensis* é conhecida como “Margaridinha”, “Maria-Mole”, “Flor das Almas” e “Tasneirinha”, no Brasil, é freqüentemente encontrada em pastagens nos estados do Rio Grande do Sul, Minas Gerais e São Paulo (CORRÊA, 1984).



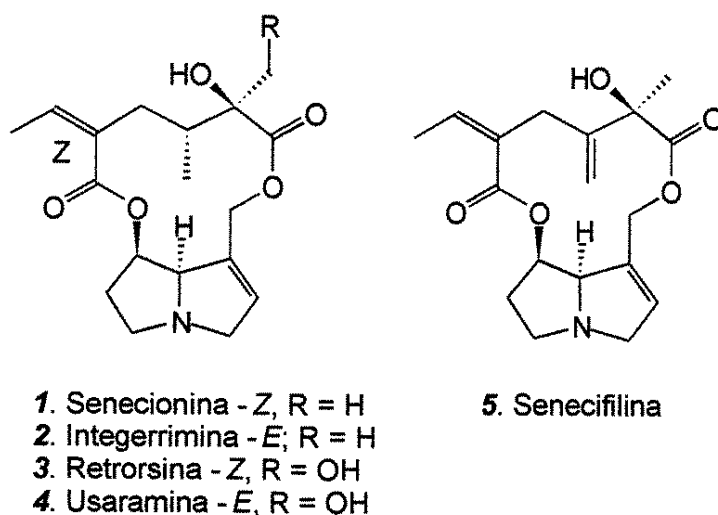
**Figura 1:** Campo contendo *Senecio brasiliensis*



*Senecio brasiliensis* é uma planta herbácea, perene, ereta e bastante ramificada com caule liso e estriado. Atinge 1-2 m de altura. Possui folhas alternadas, pinadas e profundamente lobadas até a nervura central. A parte ventral da planta é lisa, enquanto a parte superior é pilosa. As folhas são densamente agrupadas no ápice. As inflorescências são capítulos numerosos arranjados em corimbos densos com flores amareladas, que são de dois tipos: folhas em discos que possuem partes masculinas e femininas na mesma flor, e folhas radiadas que são exclusivamente femininas. O fruto é pequeno e possui plumagem branca que ajuda na dispersão pelo vento (PIO CORRÊA, 1984).

Estudos fitoquímicos realizados a partir de folhas e flores desta planta relatam a presença de diversos compostos químicos, destacando-se aqui, os alcalóides pirrolizidínicos (PAs). Os PAs são comumente encontrados em plantas pertencentes às famílias Boraginaceae, Orchidaceae, Leguminosae e Asteraceae. São compostos orgânicos cíclicos que contêm um nitrogênio (com estado de oxidação negativo), uma dupla ligação nas posições 1-2 do anel e grupos hidroxilas esterificados nas posições 7 e 9 dos carbonos (TRIGO et al., 1996).

Para avaliação da atividade antiulcerogênica, neste trabalho, foram obtidos os PAs integerrimina (2), retrorsina (3); senecionina (1), usaramina (4) e senecifilina (5), na proporção (4:86:7:1:2) (Figura 2).



**Figura 2:** Estrutura química dos Alcalóides Pirrolizidínicos

Em uma revisão foram analisadas as moléculas utilizadas para o tratamento das úlceras gástricas, LEWIS (1991), demonstra que a atividade antiulcerogênica não se limita a apenas uma classe de compostos, mas, que os compostos obtidos de plantas, com essa atividade, apresentam os mais variados tipos de estruturas químicas; do mesmo modo, diferentes mecanismos de ação têm sido propostos para estes compostos. Dentre as principais classes de substâncias com atividade antiulcerogênica encontram-se terpenos, flavonóides, glicosídeos, saponinas, polissacarídeos e alcalóides.

Embora muitos alcalóides sejam considerados extremamente tóxicos, quando usados em doses adequadas, tornam-se drogas extremamente úteis na terapia clínica. A atropina, por exemplo, um alcalóide obtido a partir da *Atropa Belladonna*, é uma droga que promove redução da secreção ácida-gástrica, tendo sido extremamente útil na terapia e no auxílio à pesquisa de novos fármacos para o tratamento das úlceras gástricas (LEWIS, 1992).

Em geral, os compostos de origem vegetal, com atividade antiulcera exercem seu efeito estimulando os fatores de proteção da mucosa gástrica, como, por exemplo, aumentando a síntese de prostaglandinas e/ou estimulando a secreção de muco e de bicarbonato. Outro importante mecanismo, estimulado por substâncias de origem vegetal, seria a inibição da secreção ácida gástrica através da interação com diferentes tipos de receptores farmacológicos, com enzimas ou hormônios, envolvidos com o processo secretor (BORRELLI e IZZO, 2000).

Embora as plantas medicinais raramente sejam utilizadas pela medicina tradicional como plantas antioxidantes, grande parte de suas propriedades terapêuticas pode também estar relacionada à sua capacidade “varredoura” de radicais livres derivados do oxigênio, ou propriedade antioxidante. Sabe-se que muitos destes radicais livres podem estar envolvidos no processo fisiopatológico de diversas patologias, inclusive as úlceras pépticas (DESMARCHELIER et al., 1999). Além disso, com o grande avanço nas pesquisas relacionadas à participação do EGF no processo de cicatrização das lesões gastroduodenais, há ainda tendência em ampliar os estudos científicos envolvendo substâncias obtidas a partir das plantas medicinais, com comprovada atividade antiulcerogênica, e a ação delas sobre a síntese de EGF e, conseqüentemente, sobre a cicatrização das lesões ulcerogênicas.

Assim, para o estudo da atividade farmacológica antiulcerogênica (preventiva ou curativa) e de seu possível mecanismo de ação, de compostos de origem vegetal ou não, foram desenvolvidos diversos modelos experimentais que possibilitam estudar o efeito de substâncias sobre distúrbios gastrointestinais. Os modelos disponíveis de indução experimental de úlceras em animais são múltiplos e podem ser conduzidos de diferentes formas, utilizando diversos agentes indutores. Estes modelos são freqüentemente na pesquisa de novos agentes, já que é possível garantir que eles reproduzem, em animais, as mesmas lesões estabelecidas em humanos.

Este trabalho teve, portanto, como objetivo avaliar a atividade antiulcerogênica de PAs obtidos a partir do extrato etanólico de flores e folhas de *Senecio brasiliensis* definindo ambas doses tóxicas e terapêuticas, bem como o(s) mecanismo(s) de ação relacionados a esta atividade farmacológica. Os resultados obtidos concluem que os PAs obtidos a partir de *S. brasiliensis* são um fonte alternativa para a indústria farmacêutica na busca de compostos farmacologicamente ativos no tratamento das úlceras pépticas.

## **2. ARTIGOS**

## **2.1- Artigo I**

### **2.1.1- Objetivos do artigo I:**

- A) Avaliar a atividade antiulcerogênica gástrica e duodenal da fração alcaloidal (PAs) obtida a partir do extrato etanólico das folhas e flores de *Senecio brasiliensis*;
- B) Definir a dose tóxica ( $DL_{50}$ ) e terapêutica de PAs, no sentido de determinar o índice terapêutico;
- C) Definir o provável mecanismo antiúlcera citoprotetor de PAs na atividade antiulcerogênica evidenciada.

### **2.1.2- Título do Artigo:**

Pharmacological mechanisms implicated in the preventive activity of pyrrolizidine alkaloids from *Senecio brasiliensis* (Asteraceae) on gastric and duodenal induced ulcer on mice and rats.

### **2.1.3- Revista Submetida:**

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#### **2.1.4- Correpondência relativa ao artigo I**

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## 2.1.5- Artigo I



**Regular Article**

**Pharmacological mechanisms implicated in the preventive activity of  
pyrrolizidine alkaloids from *Senecio Braziliensis* (Asteraceae) on gastric and duodenal  
ulcer induced on mice and rats**

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## Abstract

Pyrrrolizidine alkaloids have been identified in traditional medicinal plants of South America and Asia. We obtained the alkaloidal fraction extract (PAs) from *Senecio Braziliensis* inflorescences, containing a mixture of senecionine, interregimine, retrorsine, usaramine and seneciophylline (4:86:7:1:2); and evaluated the preventive antiulcerogenic effects of this (PAs) on standard rodent models of induced gastric and duodenal ulcers. In the HCl / ethanol-solution-induced gastric ulcer, we evaluated the therapeutic doses for PAs (6.25, 12.5, 25 and 50 mg/kg, *p.o.*) and obtained significantly reduction of gastric lesion index ( $p<0.001$ ) at three different doses (12.5, 25 and 50 mg/kg, *p.o.*). In indomethacin-bethanechol induced gastric ulcer, the PAs (12.5 mg/kg, *p.o.*) showed significant activity ( $p<0.001$ ). In hypothermic-restraint-induced gastric ulcer, the ulcerative lesion was inhibited by 54.8 % ( $p<0.001$ ). In the pylorus-ligature, PAs (12.5 mg/kg, *i.d.*), significantly increase the gastric juice content, increased the pH values and decreased the acid output. In the cysteamine induced duodenal ulcers, PAs (12.5 mg/kg, *p.o.*) showed significant inhibition ( $p<0.001$ ) of duodenal lesions (61.4 %) when compared to the respective control value. The levels of the somatostatin hormone in the blood samples of the animals pre-treated with the PAs (12.5 mg/kg) also showed increase (41.1 pmol/L) ( $p<0.001$ ). The free mucous and prostaglandin synthesis also was increases (15.0 and 13.1 %, respectively) after administration of PAs extract (12.5 mg/kg, *p.o.*). These results suggested that the PAs extract obtained from *Senecio Braziliensis* inflorescences present a significant anti-ulcer effect when assessed in these induced ulcer models. The mechanism involved with the action of the PAs extract are the cytoprotective mechanism but news assays are in progress to determine others possible mechanisms involved with the actuating of the PAs as an anti ulcer agent.

Key words: *Senecio Braziliensis*; pyrrolizidine alkaloids; antiulcerogenic activity

## Introduction

Duodenal and gastric ulcers and gastric cancer are common and serious diseases in the world population [1]. This pathology is among the most important causes of morbidity in the world population, and is common afflictions in South America.

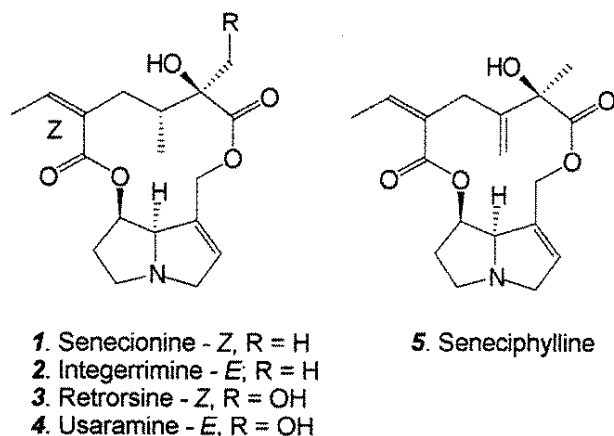
The etiology of gastroduodenal ulcers is influenced by various aggressive and defensive factors such as acid-pepsin secretion, parietal cell, mucosal barrier, mucus secretion, blood flow, cellular regeneration and endogenous protective agents (prostaglandins and epidermic growth factors) [2].

Many pharmaceutical products have been employed for the treatment of gastroduodenal ulcer and peptic disease, but these pharmaceutical products are too expensive [3]. Moreover, any pharmaceutical treatment forgets a cicatrization of the lesions with the quality and consequently the rescidive of these lesions are a common factor. Thus, in the foreseeable future, therapy for gastric and duodenal ulcers will continue being one of the major research goals.

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants [4]. In traditional medicine, several plants and herbs have been used to treat gastrointestinal disorders, including gastric and peptic ulcers [5].

*Senecio Braziliensis*, popularly known as "Flor das Almas", "Tasneirinha", "Margaridinha" or "Maria-Mole", is a native species from South America found in south and southeast Brazil, blooming in October and November [6, 7]. The leaves and inflorescences of *Senecio Braziliensis* are utilized by traditional medicine for the treatment of inflammatory processes as a regulator of the circulatory blood. Moreover, this plant is also used in folk medicine for the treatment of stomach pain [8].

It was reported that *Senecio Braziliensis* contains pyrrolizidine alkaloids (PAs) [9, 10]. These PAs are twelve membered 1,2-unsaturated macrocyclic diesters, primarily integerrimine (2), retrorsine (3); as well as their respective geometrical isomers, senecionine (1), usaramine (4) and seneciophylline (5) (Figure 1).



**Figure 1. Chemical structures of pyrrolizidine alkaloids isolated from *Senecio Braziliensis* inflorescences.**

An examination of the phytochemical literature for anti-ulcer molecules reveals that anti-ulcer activity is not confined to one class of compounds. Among the various classes of compounds are the alkaloids. Although many alkaloids are poisons, when used responsibly they are extremely useful drugs [11]. Moreover, pyrrolizidine alkaloids can displace specific ligands of the muscarinic receptors of acetylcholine, a neurotransmitter related to the ulcerogenic process [12].

In this study we prepared the alkaloidal extract (PAs) from inflorescences of *Senecio Braziliensis* which contain twelve membered 1,2-unsaturated macrocyclic pyrrolizidine diesters, and evaluated its preventive antiulcerogenic effects when administered by the oral and intraduodenal route in standard rodent models of induced gastric ulcer.

## **Material and methods**

### ***Drugs and chemicals***

Cimetidine, cysteamine, lansoprazole, indomethacin and bethanechol chloride, acetic acid, absolute ethanol, sodium bicarbonate and sodium chloride were obtained from Sigma Chemical Co. (St Louis, Mo). The PAs extract from *Senecio Braziliensis* was

dissolved in 0.9% saline solution and administered at the doses 6.25, 12.5, 25 and 50 mg/kg *p.o.* or intraduodenally depending on each experiment. The substances, reagents and extract were freshly prepared just before use.

#### ***Alkaloidal Extract Preparation***

The inflorescences of *Senecio Braziliensis* var *Braziliensis* (Spreng.) Less. Were collected in Serra do Japí, Jundiaí, SP, in October 1999, and the exsiccate deposited in the Herbarium of the Department of Zoology, Institute of Biology, UNICAMP (Voucher number 2114). The pyrrolizidine alkaloids from *Senecio Braziliensis* were extracted and characterized by GC-MS as described by Trigo et al. 1996 [13].

#### ***Animals***

The experiments were performed on male Swiss mice (30-50 g) and rats (180-250 g) obtained from the Animal House of CEMIB, UNICAMP, in Campinas, SP, Brazil. The animals were fed a certified Nuvilab CR-a diet (Nuvital<sup>®</sup>) with free access to tap water, and were housed on a 12h light/dark cycle at a humidity of 50% and a temperature of  $24 \pm 1^\circ\text{C}$ . All experiments were done in the morning. The experimental protocols were approved by the Animal Use and Care Committee of UNICAMP and were conducted in accordance with the recommendations of the Canadian Council on Animal Care and with the ethical guidelines for investigations of experimental pain in conscious animals [14].

#### **Pharmacological assays**

##### ***Nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers in cholinomimetic-treated mice***

The experiment was performed by the method of Rainsford, 1978 [15]. In this model, gastric lesions were induced using indomethacin (30 mg/kg, *s.c.*) and bethanechol (5 mg/kg, *i.p.*), administered to mice after a 24h fast. The PAs extract from *Senecio Braziliensis* (12.5 mg/kg), cimetidine (100 mg/kg) or 0.9% saline (10 ml/kg) were administered orally 30 min before the induction of gastric lesions. The animals were killed by cervical dislocation 4 h after treatment with the ulcerogenic agents and the stomachs

were removed and inflated with 4 % formalin in buffered saline. Gastric damage was determined as described by Szelenyi and Thiemer, 1978 [16].

#### ***Hypothermic restraint-stress ulcer***

The experiment was performed by the method of Levine, 1971 [17], with some modifications. After 24 h of starvation, the animals received an oral administration of the PAs extract from *Senecio Braziliensis* (12.5 mg/kg), cimetidine (100 mg/kg) or 0.9% saline (10 ml/kg). One hour after treatment, mice were immobilized in a restraint cage at 4°C for 3 h to induced gastric ulceration. The animals were killed by cervical dislocation and the stomach removed and opened along the greater curvature to determine the lesion index according Szelenyi and Thiemer, 1978 [16].

#### ***Determination of Gastric Secretion***

The assay was performed by the method of Shay, 1945 [18] with some modifications. All groups of mice fasted for 24 h, with free access to water. Immediately after pylorus ligation, the PAs extract (12.5 mg/kg), cimetidine (100 mg/kg) used as positive control, or the vehicle, 0.9% saline (10 ml/kg) was administered by intraduodenal route (*i.d.*). The animals were killed 4 h later by cervical dislocation, the abdomen opened and another ligation placed around the esophagus close to the diaphragm. The stomachs were removed and gastric juice ( $\mu$ l) and pH determined. Distilled water (5 ml) was added and the solution was centrifuged at 3000 rpm for 10 min. The total acid in the gastric secretion was determined in the supernatant volume by titration to pH 7.0 with 0.01 N NaOH. The lesion index was also evaluated in all the stomachs.

#### ***HCl/ethanol-induced ulcer***

The antiulcerogenic activity of the PAs extract was assessed as described by Mizui and Doteuchi, 1983 [19]. Mice were divided into 6 groups and fasted for 24 h prior to receiving an oral dose of the vehicle, 0.9% saline (10 ml/kg), lansoprazole (30 mg/kg) or the PAs extract (6.25, 12.5, 25 and 50 mg/kg). Fifty min after the treatments, all animals received 0.2 ml of a 0.3 M HCl / 60 % ethanol solution orally and killed 1 h later. The

stomachs were excised and inflated by injection of saline (2 ml) and opened along the greater curvature. The stomach was fixed in 5 % formalin for 30 min and gastric damage determined as described Szelenyi and Thiemer, 1978 [16].

#### ***Cysteamine-induced duodenal ulcers***

Acute duodenal lesions were induced in rats according to the method of Szabo, 1978 [20]. Two doses of cysteamine HCl (400 mg/kg) in 1 ml of distilled water were administered separately by a 4 h interval. A test sample (PAs extract, 12.5 mg/kg) was given orally 1 h prior to cysteamine administration. A control group received only the vehicle and another received cimetidine (100 mg/kg). All animals were killed 48 h after the doses of cysteamine and the duodenal lesions were examined with a 10 X magnifying binocular microscope for the presence of lesions. The area (square mm<sup>2</sup>) of each lesion was calculated by multiplying the two greatest perpendicular axis and the sum of the areas of all duodenal lesions were used as the lesion index [21].

#### ***Ethanol- induced gastric ulcer***

Gastric lesions were induced by *p.o.* administration of 1 ml of absolute ethanol per rat [22]. Test substances 0.9% saline (10 ml/kg), lansoprazole (30 mg/kg) and PAs extract (12.5 mg/kg) were given *p.o.* 30 min before the ulcerative agent. One hour after the ethanol was administered, blood was collected by abdominal aorta bleeding into tubes containing EDTA. Plasma was separated and stored at -20° C until analysis for somatostatin levels.

#### ***Plasma somatostatin levels***

Blood samples were centrifuged and the serum was stored at -20° C until assayed. Concentrations of somatostatin were determined in the plasma by means of specific radioimmunoassay [23]. Results were expressed as pmol/L of somatostatin.

#### ***Determination of Mucous in Gastric Content***

This assay was performed according to the methodology described previously by Sun et al. 1991[24], with some modifications. Mice were fasted for 24 h, under anesthesia, the abdomen incised and the pylorus ligated. The PAs extract (12.5 mg/kg body weight

(kg)) of *Senecio Braziliensis* or vehicle were administered intraduodenally after the pylorus ligation. The animals were killed by cervical dislocation 4 h after the drug treatments. The stomach content was immersed in 10 ml 0.02% alcian blue in 0.16 M sucrose / 0.05 M sodium acetate, pH 5.8, and incubated for 24 h at 20° C. The alcian blue binding extract was centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 615 nm using a light spectrophotometer U/2000 (Hitachi, Japan). The free mucous in the gastric content was calculated from the amount of alcian blue binding [mg/wet tissue (g)].

#### ***Determination of Prostaglandin Synthesis***

The assay was performed by the method of Curtis et al. 1995 [25]. Thirty minutes after treatment with the vehicle, indomethacin (20 mg/kg, *s.c.*) and PAs (12.5 mg/kg, *p.o.*), the rats were killed by cervical dislocation and the abdomen opened. The Sham group without treatment experienced the general conditions of the experimental group. Samples of the corpus (full thickness) were excised, weighed and suspended in 1 ml of 10 mM sodium phosphate buffer, pH 7.4. The tissue was minced finely with scissors, and incubated at 37° C for 20 min. Prostaglandin in the buffer was measured using an enzyme immunoassay (RPN222-Amersham).

#### ***Statistical analysis***

The results are presented as mean  $\pm$  SD. Statistical significance was determined by one-way analysis of variance followed by Dunnet's test, with the level of significance set at  $p < 0.05$ .

#### ***Results and Discussion***

Medicinal plants have been traditionally used in folk medicine throughout the world for controlling various diseases, including the peptic ulcer pathology [4]. We evaluated the antiulcerogenic activity of the PAs fraction obtained from *Senecio Braziliensis* inflorescences, using the different standard experimental models of induced gastric ulcer.

The PAs are very known by their hepatotoxicity. Most hepatotoxic PAs owe their hepatotoxicity to the presence of a 1,2-double bond in the pyrrolizidine ring and the



sterification of hydroxyl groups [26]. However, PAs can displace specific ligands of the muscarinic receptors of acetylcholine, a neurotransmitter related to the ulcerogenic process [12].

We first evaluated the toxicity of this alkaloidal fraction (PAs) *in vivo*, and obtained the  $DL_{50}$  values = 234.4 mg/kg, when administered by oral route (Data not showed). Moreover, we also evaluated the therapeutic doses of the PAs in the standard rodent models of induced gastric ulcer. This parameter were evaluated in the HCl / ethanol solution administration.

Oral administration of the HCl / ethanol solution to the mice clearly produced the expected characteristic zonal necrotizing mucosal lesions. This damage may be due to a direct action of the gastric epithelium causing lipid peroxidation. Ethanol treatment induces intracellular oxidative stress and produces mitochondrial permeability transition and mitochondrial depolarization, which precede cell death in gastric mucosal cells [27]. We studied the preventive antiulcerogenic activity of PAs using four doses (6.25, 12.5, 25 and 50 mg/kg) in the HCl-ethanol-induced gastric ulcer model.

The ulcerative lesion index was significantly reduced by the PAs extract at three tested doses (12.5, 25 and 50 mg/kg) with the inhibition of the lesions of (32.9, 42.5 and 66.8 %, respectively). The dose of 6.25 mg/kg not showed significant results, but reduced the ulcerative lesion index in the 8.9% (Table 1). With this results, we determined 12.5 mg/kg with the parameter doses utilized in the others experiments.

Table 1: Effects of lansoprazole and four different doses of PAs extract from *Senecio Braziliensis* on HCl/ethanol-induced gastric ulcer in mice.

Treatments ( <i>p.o.</i> )	Dose (mg/kg)	<i>n</i>	ULI	Inhibition (%)
0.9 % Saline (control)	10 ml/kg	8	28.0 ± 1.4	-
Lansoprazole	30	8	11.0 ± 4.2 **	60.7
PAs	6.25	8	25.5 ± 1.8	8.9
	12.5	8	18.8 ± 1.5 **	32.9
	25	8	16.1 ± 1.2 **	42.5
	50	8	9.3 ± 0.7 **	66.8

Data are presented as the mean ± SD (*n*=8). ANOVA following F values:  $F_{(5,42)}=101.3$  ( $p<0.05$ ). Dunnett's test \*\* $p<0.01$ .

Prostaglandins (PGs) play an important physiological role in maintaining the integrity of the gastric mucosa. NSAIDs acting in the inhibition of cyclooxygenase (COX), the key enzyme in PG formation induces gastric mucosal injury in rodents and humans [28]. The co-administration of cholinomimetic agents such as bethanechol promotes a synergism with NSAIDs in the gastric lesion induced by increase in the secretion of acid and pepsin in the stomach [15]. Exogenous PGs protect the gastric mucosa against various types of damage caused by necrotizing agents, including gastric ulcers. In addition, PGs are known to play an important role in the healing of gastric ulcers. PGs of the E series have an inhibitory action on acid output and maintain the integrity of the gastric mucosa [29].

The PAs extract showed significant preventive activity (44.4 %) when evaluated in NSAID / cholinomimetic-induced gastric ulcer compared to the respective control value (Table 2). These results suggest the possible involvement of prostaglandins in the cytoprotection / antiulcer effect of the extract.

Table 2: Effects of PAs extracts from *Senecio Braziliensis* administered orally on indomethacin-bethanechol-induced gastric ulcer in mice.

Treatments ( <i>p.o.</i> )	Dose (mg/kg)	<i>n</i>	ULI	Inhibition (%)
0.9 % Saline (control)	10 ml/kg	8	9.0 ± 0.8	-
Cimetidine	100	8	3.9 ± 0.6 **	56.7
PAs	12.5	8	5.0 ± 1.7 **	44.4

Data are presented as mean ± SD (*n*=8). ANOVA following F values:  $F_{(2,21)}=44.442$  ( $p<0.05$ ). Dunnett's test : \*\*  $p<0.01$ .

Stress-induced ulcers are probably mediated by histamine release with enhancement in acid secretion and a reduction in mucous production. Moreover, stress-induced ulcers can be prevented partially or entirely by vagotomy; vagal over-activity has been suggested to be the principal factor in stress-induced ulceration [28].

In this model the PAs extract also significantly suppressed the development of ulcers ( $p<0.01$ ) when compared to the respective control (Table 3). These results could be due to its antihistaminic, anticholinergic and antisecretory effects.

Table 3: Effects of PAs extracts from *Senecio Braziliensis* administered orally for hypothermic restraint gastric ulcer induced in mice.

Treatments ( <i>p.o.</i> )	Dose (mg/kg)	<i>n</i>	ULI	Inhibition (%)
0.9 %Saline (control)	10 ml/kg	8	7.3 ± 1.2	-
Cimetidine	100	8	3.0 ± 0.8 **	58.9
PAs	12.5	8	3.3 ± 1.0 **	54.8

Data are presented as the mean ± SD (*n*=8). ANOVA following F values:  $F_{(2,21)}=44.318$  ( $p<0.05$ ). Dunnett's test : \*\* $p<0.01$ .

Pylorus ligation is an important procedure that shows the possible changes of biochemical parameters of gastric content after the various treatments. The PAs extract

caused significant changes in all parameters evaluated when administered by intraduodenal route at the dose of 12.5 mg/kg (Table 4). A significant increase in gastric fluid volume (191.8  $\mu$ l), a decrease in acid output (20.9  $\mu$ Eq/4 h) with an elevation in gastric pH (5.0) were observed after intraduodenal administration of the PAs extracts when compared to the respective control values (118.4  $\mu$ l, 25.3  $\mu$ Eq/4 h and 3.2, respectively). These results showed that the antiulcerogenic activity of this extract was not related only to a local neutralization of gastric content but also had this effect after the absorption of PAs or a systemic effect.

Table 4: Effects of PAs extract from *Senecio Braziliensis* administered intraduodenally (*i.d.*) on the biochemical parameters of gastric juice obtained from mice with pylorus ligation.

Treatments ( <i>i.d.</i> )	Dose (mg/kg)	<i>n</i>	pH (Units)	Volume gastric Juice ( $\mu$ l)	Total Acid Output ( $\mu$ Eq/4h)
0.9 % Saline (control)	10 ml/kg	8	3.2 $\pm$ 0.8	118.4 $\pm$ 25.1	25.3 $\pm$ 2.0
Cimetidine	100	8	4.9 $\pm$ 1.1 **	101.3 $\pm$ 10.5	19.5 $\pm$ 1.7 **
PAs	12.5	8	5.0 $\pm$ 1.1 **	191.8 $\pm$ 30.6 **	20.9 $\pm$ 0.9 **

Data are presented as the mean  $\pm$  SD ( $n=8$ ). ANOVA for pH values:  $F_{(2,21)}=8.026$ ; ;  $F_{(2,21)}=33.091$  for gastric juice;  $F_{(2,16)}=17.184$  for acid output and  $F_{(2,21)}=28.551$  ( $p<0.05$ ).

Dunnett's Test: \*\* $p<0.01$ .

Cysteamine-induced duodenal ulcer in the rat is widely used as a model of peptic ulcer disease. This chemically induced ulcer resembles the duodenal ulcer in man [29]. Cysteamine induces long-lasting hypersecretion of gastric acid, which may be partly due to increased plasma levels of gastrin. In addition, cysteamine inhibits secretion of alkaline mucus from the duodenal Brunner's gland. Hypersecretion of acid, disturbed gastroduodenal motility, hypergastrinemia and decreased mucosal resistance have all been implicated in the pathogenesis of duodenal ulcer disease in man [30].

We also evaluated the preventive antiulcerogenic activity of the PAs in the duodenal ulcer induced by cysteamine administration in rats (Table 5). PAs showed significant activity ( $p<0.05$ ) when compared to the respective control, with 61.4 % inhibition of duodenal lesions. The development of duodenal ulcers in response to cysteamine is inhibited by anticholinergic agents, antacids, prostaglandins and histamine H<sub>2</sub>-receptor antagonists [31]. Therefore, the inhibition of gastric acid secretion and mucus and prostaglandin release and/or gastric acid neutralization and a mechanical barrier occurring in the duodenum may to interfere with duodenal mucosa protection [32].

Table 5: Effects of PAs extract from *Senecio Braziliensis* on cysteamine-induced duodenal ulcer in rats.

Treatments ( <i>p.o.</i> )	Dose (mg/kg)	<i>n</i>	Ulcerative Index (mm <sup>2</sup> )	Inhibition (%)
0.9% Saline (control)	10 ml/kg	7	10.1 ± 1.3	-
Cimetidine	100	7	3.3 ± 0.8 **	67.3
PAs	12.5	7	3.9 ± 1.1 **	61.4

Data are presented as the mean ± SD ( $n=7$ ). ANOVA following F values:

$F_{(2,18)}=85.961$  ( $p<0.05$ ). Dunnett's test \*\* $p<0.001$ .

Actually several works showed the involvement of the gastrointestinal hormones gastrin, somatostatin (SMT) and cholecystokinin (CCK) on the pathophysiology of peptic ulcers disease. Somatostatin, a cyclic tetradecapeptide, is widely distributed throughout the gastrointestinal tract. It is mainly confined to D cells in the mucosal layer of the stomach and pancreas, minute amounts being found in neural cells along with other neuropeptides [33]. In the stomach somatostatin serves as a paracrine regulator of both acid and gastrin release, exerting some of its actions via interference in cAMP pathways [34]. Other effects of somatostatin in the stomach include inhibition of pepsinogen secretion, gastric emptying and stimulation of gastric mucus output [35]. Moreover, László et al., 1989 [36], related that the administration of somatostatin prevented cysteamine-induced duodenal ulcer, as well as ethanol-induced hemorrhagic gastritis in rats.

Table 6: Effects of PAs extracts from *Senecio Braziliensis* administered orally for Ethanol induced ulcer in the somatostatin plasma levels.

Treatments ( <i>p.o.</i> )	Dose (mg/kg)	<i>n</i>	Somatostatin plasma levels (pmol/L)
0.9 %Saline (control)	10 ml/kg	8	13.3 ± 5.7
Sham	-	8	8.3 ± 2.1
Lansoprazole	30	8	101.7 ± 17.0 **
PAs	12.5	8	41.1 ± 3.5 **

Data are presented as the mean ± SD (*n*=8). ANOVA following F values:

$F_{(3,24)}=152.39$  ( $p<0.05$ ). Dunnett's test : \*\*  $p<0.001$ .

Our experiments showed that the PAs fraction at doses of 12.5 mg/kg on gastric ulcer induced by administration of ethanol in rats promoted the increase ( $p<0.01$ ) of plasma levels of somatostatin when compared to the respective control values (Table 6).

Finally, we investigated free mucous production and prostaglandin synthesis after the administration of the PAs extracts (12.5 mg/kg, *p.o.*), of *Senecio Braziliensis* for a possible mechanism for the increase in mucosal protective factors.

We can observed that PAs extract showed a significantly increase of free mucous (15 %) in relation to the respective control value (Table 7) and increased the synthesis of prostaglandin levels (13%) (Table 8).

Table 7: Effect of PAs Extract from *Senecio Braziliensis* Administered by the Intraduodenal Route on Alcian Blue Binding to Free Gastric Mucous from Pylorus/Ligature Mice.

Treatments (i.d.)	Dose (mg/kg)	n	Alcian blue Bound [mg/wet tissue (g)]	Alcian blue bound (%)
0.9 % Saline (control)	10 ml/kg	8	1.98 ± 0.1	-
Sham	-	8	2.0 ± 0.1	-
Indomethacin	20	8	1.60 ± 0.2 **	-20.0
PAs	12.5	8	2.30 ± 0.3 **	15.0

Data are presented as the mean ± SD (n=8). ANOVA following F values:  $F_{(2,28)} = 20.446$  ( $p < 0.05$ ) for alcian blue bound. Dunnett's test: \*\* $p < 0.01$ .

Table 8: Effects of PAs Extract from *Senecio Braziliensis* on Prostaglandin Synthesis by the Gastric Mucosa of Rats.

Treatments (p.o.)	Dose (mg/kg)	n	Prostaglandin synthesis (pg/mg tissue)	Increase (%)
0.9% Saline (p.o.) (control)	10 ml/Kg	8	54.0 ± 1.4	-
Sham	-	8	54.4 ± 3.4	-
Indomethacin (s.c.)	20	8	41.5 ± 4.4 **	-23.1
PAs (p.o.)	12.5	8	61.1 ± 5.5 **	13.1

Data are presented as the mean ± SD (n=8). ANOVA following F values:  $F_{(3,28)} = 33.554$  ( $p < 0.05$ ). Dunnett's test: \*\* $p < 0.001$ .

Karadi et al. 1999 [37], proved that antimuscarinic drug such as atropine showed a cytoprotective effect on indomethacin induced gastric ulcer and this activity disappears in the acute phase of surgical vagotomy. Considering that PAs can displace specific ligands of the muscarinic receptors of acetylcholine, a neurotransmitter related to the ulcerogenic process [13], probably the cytoprotection showed by this extract involves the participation

of vagal nerve. Moreover, the increase of somatostatin levels can be contributed to the increase of the cytoprotective factors such as mucous and prostaglandins.

In conclusion, the PAs extract obtained from *Senecio Braziliensis* inflorescences is a potent antiulcerogenic compound with cytoprotective activity on gastric and duodenal ulcer. However, the antisecretory mechanism is still possible in this activity. These compounds may yield new antiulcerogenic drugs; others studies to investigate their mechanisms of action are now in progress.

### **Acknowledgments**

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### 2.1.6- Resumo do artigo I

*Senecio brasiliensis*, uma planta nativa da América do Sul e popularmente conhecida como “Margaridinha”, “Flor das Almas”, “Tasneirinha” é comumente encontrada nos estados das regiões Sul e Sudeste do Brasil. Alguns trabalhos relatam a utilização das folhas e flores de *Senecio brasiliensis* para o tratamento de moléstias do trato gastrointestinal, inclusive úlceras pépticas. Além disso, diversos estudos fitoquímicos relatam a presença nos extratos obtidos a partir desta planta de alcalóides pirrolizidínicos (PAs).

Dentre os diversos compostos com provável atividade antiulcerogênica encontram-se os alcalóides. Com base nestes dados, este primeiro trabalho teve como objetivo avaliar a provável atividade antiulcerogênica gástrica e duodenal dos PAs obtidos a partir do extrato etanólico das folhas e flores de *Senecio brasiliensis*. Os modelos padrões de indução de úlceras gástrica e duodenal em roedores e que mimetizam esta patologia na raça humana e quais suas doses tóxica e terapêutica.

Foram analisados ainda o(s) provável(eis) mecanismo(s) de ação envolvidos nesta atividade antiulcerogênica detectada através de vias citoprotetoras da mucosa gástrica (prostaglandinas e muco) e também da participação do hormônio somatostatina relacionado ao processo de redução da secreção ácida-gástrica.

Os resultados demonstraram que os PAs apresentam atividade antiulcerogênica gástrica e duodenal em uma dose (12.5 mg/Kg) quase 20 vezes menor que a tóxica (234.4 mg/Kg), além de apresentar mecanismos citoprotetores envolvendo aumento da produção de muco, prostaglanina e somatostatina.

## **2.2- Artigo II**

### **2.2.1- Objetivos do artigo 2**

- A) Avaliar a atividade antiulcerogênica aguda e crônica da fração alcaloidal de PAs em ratos
- B) Analisar o envolvimento dos PAs com mecanismos de cicatrização de lesões gástricas como níveis plasmáticos de gastrina e expressão, a partir do RNAm do fator de crescimento epidermal (EGF)

### **2.2.2- Título do artigo:**

Modulation of gastrin and epidermal growth factor by pyrrolizidine alkaloids obtained from *Senecio brasiliensis* in the acute and chronic induced gastric ulcers

### **2.2.3- Revista Submetida: Canadian Journal of Physiology and Pharmacology**

#### **2.2.4- Correspondência Relativa ao Artigo II**

Dr. Alba R. M. Souza Brito  
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Campinas, SP  
BRAZIL

Dear Dr. Souza Brito:

MS No. 03-000133

MS Title: Modulation of gastrin and epidermal growth factor by pyrrolizidine alkaloids obtained from *Senecio brasiliensis* in the acute and chronic induced gastric ulcers

Authors: Toma W et al.

Your manuscript now been examined by two expert referees, and is not acceptable for publication in the *Canadian Journal of Physiology and Pharmacology* in its present form. The reviewers have raised several concerns, and provide suggestions for revision. Referee #1 made a number of excellent suggestions to improve the overall quality of the manuscript. Please pay particular attention to the following: 1) dose-response experiment should be performed; 2) qualitative and quantitative analysis of the main active component of the alkaloids from *Senecio brasiliensis*; and 3) results of the study must be described in the Results section. Referee #2 had similar concerns with respect to the dose used in the study in addition to the other points noted under "Specific Comments".

If you feel you can satisfactorily answer the criticisms of the referees, please send **directly to me at the address below**, three copies of the revised version of this manuscript, along with a letter in which you detail the changes you have made in response to each of the reviewers' concerns. Please also send a disk containing the final version of the manuscript with the software identified, and a second disk containing the figures drawn according to the electronic graphics instructions previously sent to you. The file of this manuscript remains open for two months, after which time any revision will be considered as a new submission. If you have not already completed it, please return the Copyright Transfer form signed by all authors.

Thank you for submitting your work to the *Canadian Journal of Physiology and Pharmacology* and I look forward to receiving your revised manuscript.

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## **2.2.5- Artigo II**

**Modulation of gastrin and epidermal growth factor by pyrrolizidine alkaloids  
obtained from *Senecio brasiliensis* in the acute and chronic induced gastric ulcers**

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## Abstract

We investigated the antiulcerogenic activity of pyrrolizidine alkaloids (PAs) integerrimine, retrorsine, senecionine, usaramine and seneciophylline, an alkaloidal extract obtained *Senecio brasiliensis*. The PAs extract demonstrated significantly activity in both, acute and chronic induced gastric ulcers on rats. The mechanisms implicated on this activity were evaluated by determination of gastrin plasma levels in rats submitted to the acute treatment with PAs extract and by expression of mRNA of Epidermal Growth Factor (EGF) after chronic treatment with this extract. The results showed that the PAs extract increased both, the levels of gastrin and the expression of EGF on these animals. Moreover, the histological examinations showed a reduction of exfoliation of superficial cells, hemorrhages and blood cell infiltration. We concluded that the PAs showed an important and qualitative antiulcerogenic activity mediated by increase of gastrin secretion and mRNA expression of EGF.

Keywords: *Senecio brasiliensis*, pyrrolizidine alkaloids, antiulcerogenic activity, gastrin, EGF



## **Introduction**

Gastric ulcers are a considerable medical problem and their complications still carry a significant mortality percentage on the world population. Several mechanisms have been implicated in the pathogenesis of gastric ulcers involving increase in gastric acid and pepsin secretion, a decrease in gastric blood flow, suppression of endogenous generation of prostaglandins, increase generation of reactive oxygen species, inhibition of mucosal growth and cell proliferation (Konturek et al., 2001).

Furthermore, several works showed the involvement of the gastrointestinal hormones such as gastrin on the pathophysiology of gastric ulcers disease (Dockray 1999) and the participation of the epidermal growth factor on the cicatrization of the ulcerogenic process (Tarnawski et al. 2001).

Gastrin is a polypeptide hormone that is synthesized in gastrin cells and has an important role in modulating various functions in the gastrointestinal tract, including acid secretion, motility, and cell proliferation (Komori et al., 2002). Furthermore, gastrin has protective action on gastric mucosa against ethanol-induced injury in rats and induces growth-promoting effects on diversity of target cells, such as epidermal growth factors (Tsuji et al., 2002).

Growth factors, such as EGF, are polypeptides that bind to cell receptors and stimulate important cellular elements of ulcer healing such as angiogenesis, granulation and tissue re-epithelization (Szabo and Vincze 2000). EGF is a 53-amino acid polypeptide synthesized mainly in the salivary glands, kidney, duodenal Brunner's glands, pancreas, liver, and lactating mammary glands. Large amounts of EGF may be found throughout the lumen of the gastrointestinal tract (Elliot et al. 2000). Actually, some studies have reported the role of the EGF as an element that increase healing rates and, mainly, that improve the quality of the ulcer healed.

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. Natural compounds can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development and the discovery of new therapeutic properties not yet attributed to known compounds (Rates, 2001).

*Senecio brasiliensis* is a native species from South America; it is found in south and southeast Brazil. The leaves and inflorescences of *Senecio brasiliensis* are used in traditional medicine for the treatment of stomach pain (Serra 1994). Popularly, this plant is known as "Flor das Almas", "Tasneirinha", "Margaridinha" or "Maria-Mole"(Pio Corrêa 1984); several works reported the presence of pyrrolizidine alkaloids (PAs) on this specie (Trigo 2000).

The aim of the present study was to evaluate the effect of PAs on the healing of ethanol and acetic acid-induced gastric ulcers in rats and on mRNA expression of EGF and levels of gastrin hormone, to determine the effect of PAs inhibition on the mucosal recovery from the gastric lesions induced.

## **Material and methods**

### ***Drugs and chemicals***

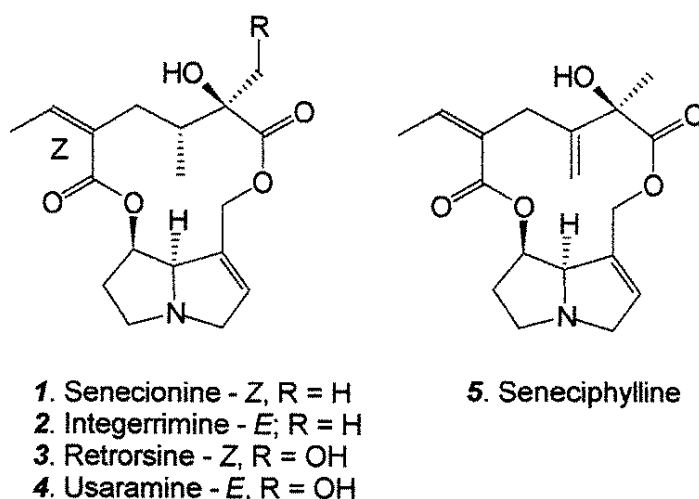
Ethanol, acetic acid and lansoprazole were obtained from Sigma Chemical Co. (St Louis, Mo). Gastrin-17 was obtained from Cis bio International. The PAs extract from *Senecio brasiliensis* was dissolved in 0.9% saline solution and administered at the dose of 12.5 mg/kg p.o. in the ethanol induced-gastric ulcer and in the acetic-acid induced gastric ulcers. The substances, reagents and extract were freshly prepared just before use.

### ***Alkaloidal Extract Preparation***

The inflorescences of *Senecio brasiliensis* var *brasiliensis* (Spreng.) Less. were collected in Serra do Japí, Jundiaí, SP, in October 1999, and the exsiccate deposited in the Herbarium of the Department of Zoology, Institute of Biology, UNICAMP (Voucher number 2114). The pyrrolizidine alkaloids (Figure 1) were extracted and characterized by GC-M, showing about 20 mg/g of dry weight of a mixture of 5% senecionine, (1) 79% integerrimine (2), 9% retrorsine (3), 6% usaramine (4), and 1% seneciphylline (5). (see Figure 1 for structures). The amount and relative abundance of these PAs are variable among populations but integerrimine, followed by retrorsine are always the main PAs (Klitzke and Trigo, 2000; Trigo et al., 2003).

### ***Animals***

All experiments were performed on male rats (180-250 g) obtained from the Animal House of CEMIB, UNICAMP, in Campinas, SP, Brazil. The animals were fed a certified Nuvilab CR-a diet (Nuvital<sup>®</sup>) with free access to tap water, and were housed on a 12h light/dark cycle at a humidity of 50% and a temperature of  $24 \pm 1^\circ\text{C}$ . All experiments were done in the morning. The experimental protocols were approved by the Animal Use and Care Committee of UNICAMP and were conducted in accordance with the recommendations of the Canadian Council on Animal Care and with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmerman 1983).



**Figure 1: Pyrrolidizidine alkaloids isolated from *Senecio brasiliensis* inflorescences**

### **Pharmacological assays**

#### *Ethanol- induced gastric ulcer*

Gastric lesions were induced by p.o. administration of 1 ml of absolute ethanol per rat (Robert et al 1979). Test substances 0.9% saline (10 ml/kg), lansoprazole (30 mg/kg) and PAs extract (12.5 mg/kg) were given p.o. 30 min before the ulcerative agent. One hour

after the ethanol was administered, blood was collected by abdominal aorta bleeding into tubes containing EDTA. Plasma was separated and stored at -20° C until analysis. The rats were sacrificed and the stomachs were removed, opened along the great curvature and then examined the ulcerative lesion index (ULI-mm<sup>2</sup>) for each stomach. All results were compared to the respective control group (0.9% Saline).

#### *Chronic induced gastric ulcer*

This experiment was performed according Takage and Okabe 1969. Rats starved for 12 hours underwent laparotomy under pentobarbital (50 mg/kg body wt). Acetic acid 30 % (50 µL) was applied to the serosa of lower corpus at the posterior wall through a polyethylene tube (4 mm ID) for 90 seconds. The serosal area was then washed with isotonic saline and the abdomen closed. Sham operated rats underwent similar procedures without acetic acid administration. During 14 days the groups of animals received 0.9% Saline (n=7), lansoprazole 30 mg/kg (n=7) and PAs extract 12.5 mg/kg (n=7), all in final volume of the 10 ml/kg by oral route. Rats were killed 15 days after operation. The stomachs were removed to determine the lesion area (mm<sup>2</sup>) and the portion of the stomach containing the oxyntic mucosa were then dissected out, weighed and stored frozen at -80°C until analysis. In some animals of each group, for histological assessment, the gastric corpus was fixed in phosphate-buffered formalin, sectioned, and paraffin-embedded. Semithin sections were deparaffinized, stained with hematoxylin and eosin, and examined under a light microscope. All results were compared to the respective control group (0.9% Saline).

#### *Plasma gastrin levels*

Blood samples were centrifuged and the serum was stored at -20° C until assayed. Concentrations of gastrin were determined in the plasma by means of specific radioimmunoassay (Mayer 1974). Results were expressed as picograms per milliliter.

#### **Molecular assay**

*Reverse-transcriptase polymerase chain reaction for detection of mRNA for EGF*

This experiment was performed according Konturek et al. 1998. Total RNA was isolated from the mucosal specimens and stored at -80°C. A rapid guanidium isothiocyanate/phenol chloroform single step extraction kit from Stratagene® was used. Following precipitation, the RNA was resuspended in Rnase-free TE buffer and the concentration was estimated by absorbance at 260 nm wave-length. The quality of each RNA preparation was determined by agarose-formaldehyde gel electrophoresis and ethidium bromide staining. RNA samples were stored frozen at -80°C until analysis. First-strand cDNA was synthesized from total cellular RNA (5 µg) using a 200 U StrataScript™ reverse transcriptase (Stratagene, La Jolla, USA) and oligo (dT) primers (Stratagene La Jolla, USA) according to standard techniques. Briefly, 5 µg of total RNA was used as the template to synthesize complementary DNA with 2.5 U of Moloney murine leukemia virus reverse transcriptase in 5 µg of buffer containing 10 mM/1 Tris-HCl, pH 8.3; 50 mM/1 KCl; 5 mM/1 MgCl<sub>2</sub>; 1mM/1 each deoxyribonucleoside triphosphates; 2.5 mM/1 of oligo (dt) primers and 1.4 U/µg de RNase block. Reverse transcription was performed at room temperature for 20 min, then at 37°C for 15 min, at 90°C for 5 min and at 5°C for 5 min. The resulting complementary DNA was used as a template for the subsequent polymerase chain reaction. The reaction mixture for the polymerase chain reaction contained cDNA template (2 µl), 50 pmol of each primer and 2.5 U of Taq DNA polymerase (Promega) in 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.5 mM dNTPs in a volume of 50 µl. Reverse transcriptase blanks (no RNA included) and polymerase chain reaction blanks (no cDNA products) were included in each analysis. Primers were synthesized by Biometra® (Göttingen, Germany). The nucleotide sequences of the rat EGF primers were based on the published cDNA sequences encoding rat preproEGF (Fan et al. 1995; Saggi et al. 1992). Rat EGF sense primer was 5'-GACAACTCCCCTAAGGCTTA-3' (nucleotides 2804-2823); the EGF antisense primer was 5'-CATGCACAGGCCACCATTGAGGCAGTACCCATCGTACGA-3' (nucleotides 3332-3370) (Saggi et al., 1992; Fan et al., 1995). Concomitantly, amplification of control rat β-actin (ClonTech, Palo Alto, CA) was performed on the same samples to assess RNA integrity. To maximize amplification specificity, a hot start polymerase chain reaction was performed in a Perkin Elmer Cetus DNA thermal cycler for 33 cycles (94°C for 1 min,

60°C for 45 s, 72°C for 2 min) using AmpliWax® PCR Gen 50 beads. Briefly, after adding primers, buffer and dNTPs, an AmpliWax polymerase chain reaction Gen was added and heated to 80°C for 10 min. Then the Taq polymerase, cDNA sample and buffer were pipetted into the PCR mixture. 8 µl aliquots of amplified polymerase chain reaction product were electrophoresed on a 1.5% agarose gel stained with ethidium broide and the visualized under UV light. The location of predicted polymerase chain reaction products (base pairs) was confirmed by using DNA digest PhiX174/HaeIII as standard size marker. The gel was then photographed under UV transillumination.

## Results

**Ethanol induced-gastric ulcers.** Table 1 shows the results obtained with experimental model of ethanol-induced acute ulcers in rats after PAs treatment. PAs extract at dose of 12.5 mg/Kg p.o., significantly decreased the ulcerative lesion index (ULI) by 66.0 % (p<0.01) when compared to the respective control value. Lansoprazole, a drug reference also demonstrated a significantly reduction of the ULI (p<0.01).

**Table 1. Effects of PAs extract obtained from *Senecio brasiliensis* administered orally on ethanol absolute-induced gastric ulcer in rats.**

Treatments (p.o.)	Dose (mg/kg)	N	UL (mm <sup>2</sup> )	Inhibition (%)
0.9% Saline	10 mL/kg	7	103 ± 12.1	-
Sham	-	7	-	-
Lansoprazole	30	7	16.0± 4.6 **	84.5
PAs	12.5	7	35 ± 9.3 **	66.0

Results are presented as mean ± SD of 7 rats in each group. ANOVA  $F_{(3,24)} = 226.33$  (p<0.05) for ULI mm<sup>2</sup>. Dunnett's test: \*\*P<0.01

**Serum Gastrin Levels.** Our results demonstrated that PAs extract produced a significantly increase of the plasma gastrin levels (192.1 µU/mL) (p<0.01), when compared to the respective control value (96.4 µU/mL) (Table 2).

**Chronic induced-gastric ulcers.** The antiulcerogenic effect of the PAs fraction (12.5 mg/kg, p.o.) on chronic induced gastric ulcer after 14 days of treatment, demonstrated significantly reduced damage of the gastric lesion by 63.2 % ( $p < 0.01$ ) when compared to the respective control value (Table 3).

**Table 2. Effects of PAs extract obtained from *Senecio brasiliensis* administered orally on gastrin plasma levels after ethanol induced gastric ulcer**

Treatments (p.o.)	Dose (mg/kg)	N	Gastrin levels $\mu\text{U/mL}$	Increase (%)
0.9% Saline	10 mL/kg	7	$96.4 \pm 7.8$	-
Sham	-	7	$66.1 \pm 4.4^{**}$	-31.4
Lansoprazole	30	7	$153.4 \pm 7.9^{**}$	59.1
PAs	12.5	7	$192.1 \pm 28.6^{**}$	99.3

Results are presented as mean  $\pm$  SD of 7 rats in each group. ANOVA  $F_{(3,24)} = 93.085$  ( $p < 0.05$ ) for gastrin levels. Dunnett's test:  $**P < 0.01$

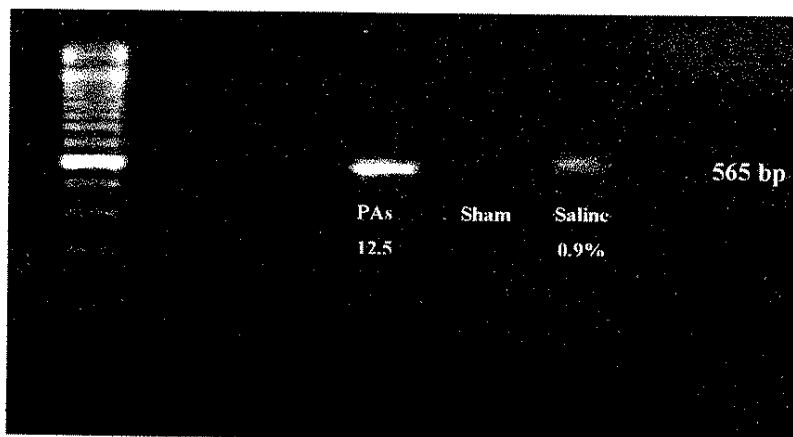
**Table 3. Effects of PAS extract from *Senecio brasiliensis* inflorescences administered orally during 14 days after acetic acid induced gastric ulcer in rats.**

Treatments (p.o.)	Dose (mg/kg)	N	ULI	Inhibition (%)
0.9% Saline	10 mL/kg	7	$28.0 \pm 5.7$	-
Sham	-	7	-	-
Lansoprazole	30	7	$12.6 \pm 4.0^{**}$	55.0
PAs	12.5	7	$10.3 \pm 5.0^{**}$	63.2

Results are presented as mean  $\pm$  SD of 7 rats in each group. ANOVA  $F_{(3,24)} = 50.946$  ( $p < 0.05$ ) for ULI  $\text{mm}^2$ . Dunnett's test:  $**p < 0.01$

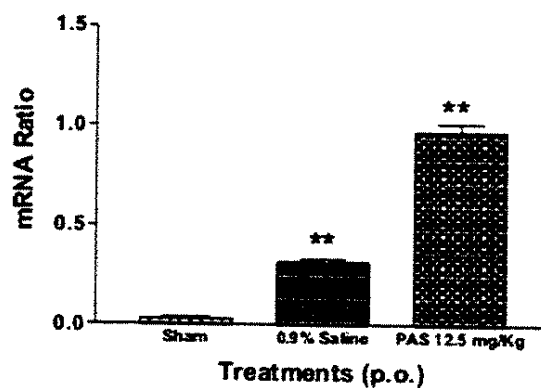
**Expression of mRNA of EGF.** The expression of EGF mRNA was detected using (RT-PCR) in gastric mucosa after chronic induced-gastric ulcers. PAs extract (12.5 mg/kg,

p.o.) after 14 day of treatment demonstrated an increase of the expression of EGF mRNA (Figure 2, 3). This result was less detectable in the intact gastric mucosa (Sham group).



**Figure 2:** Expression of mRNA of EGF after the treatment of PAs extract (12.5 mg/kg) by 14 days on acetic-acid chronic ulcer and analyzed by RT-PCR

**Figure 3.** Densiometric data Expression of mRNA of EGF after the treatment of PAs extract (12.5 mg/kg) by 14 days on acetic-acid chronic ulcer and analyzed by RT-PCR

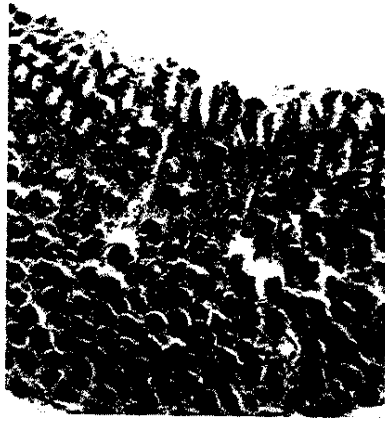


Results are presented as mean  $\pm$  SD of 7 rats in each group. ANOVA  $F_{(2,18)} = 314.3$  ( $p < 0.05$ ) for mRNA mRNA ratio expression. Dunnett's test: \*\* $p < 0.01$

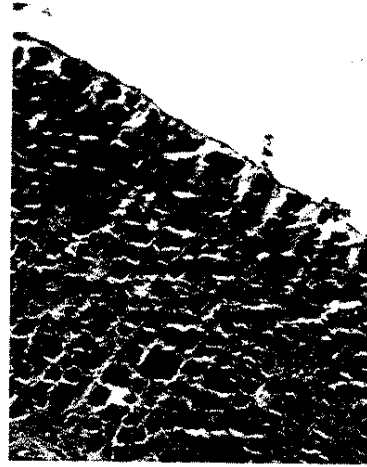
**Histological analysis.** Histologically, the damage areas of the Saline treatment group (figure 4) included exfoliation and necrosis of superficial cells, generalized blood cell infiltration and evident signs of reperfusion in comparison with non-ulcerated gastric



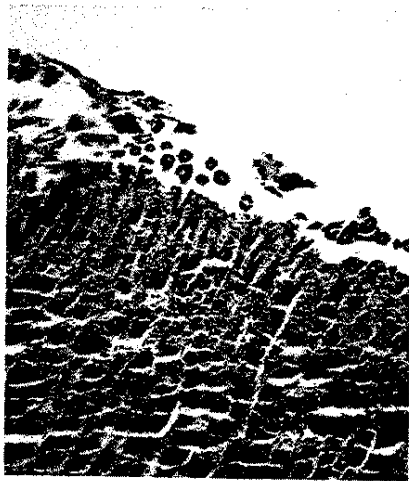
mucosa (Figure 3) and lansoprazole treatment (Figure 5). The treatment with PAs demonstrated a reduction of this aggressive factors in the mucosal of these animals (Figure 6).



**Figure 4**



**Figure 3**



**Figure 5**



**Figure 6**

### **Discussion**

Gastrointestinal disorders are one of the most important causes of morbidity in the world. Many pharmaceutical products employed for the treatment of gastroduodenal ulcers and peptic diseases, have decreased the mortality and morbidity rates, but they can produce

many adverse effects and or the recurrence of this pathology. Moreover, these pharmaceutical products are too expensive for the population (Germano et al., 1998). Szabo and Vincze 2000, showed that the healing rates with H<sub>2</sub> antagonists and with proton pump inhibitor omeprazole are about 80-100% after a 4-8 week therapy; the recurrence of ulcer, within 1 year after stopping the treatment, is between 40-80% in most of studies.

Natural products research can often be guided by ethnopharmacological knowledge, and it can make substantial contributions to drug innovation by providing novel chemical structures and/or mechanisms of action (Rates 2001).

The present study demonstrated that PAs (a naturally occurring alkaloid) protected gastric mucosa from ethanol and acetic acid-induced injury in rats.

Ethanol, when given intragastrically, induces the dissolution of mucous constituents in gastric wall, a concomitant fall in the transmucosal potential difference, increase on the fluxes of Na<sup>+</sup> and K<sup>+</sup> into to the lumen, pepsin secretion, the loss of H<sup>+</sup> ions and the release of histamine content into the lumen. Ethanol treatment also induces intracellular oxidative stress and produces mitochondrial depolarization, with preced cell death in gastric mucosal cells (Szabo 1987).

It has been found that ethanol induced ulcers are not inhibited by antisecretory agents such as cimetidine, but are inhibited by agents that enhance mucosal defensive factors such as prostaglandins (Toma et al 2002). Konturek et al. 2001, showed that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) accelerated ulcer healing via inhibition of apoptosis in the gastric epithelium.

In the digestive system, is knowed that gastrin participate in the control of gastric secretory functions. The polypeptide hormone, gastrin, is normally released by G cells of the gastric antrum in response to a protein-contains meal, to gastric distension and to increase in intragastric pH. The mainly physiological effects of gastrin are stimulation of gastric acid secretion by parietal cells in the oxyntic mucosa of the stomach and a trophic action on the gastrointestinal mucosa (Hirschowitz, 1995).

Tsuji et al., 2002, showed that the 14-day administration of lansoprazole was also associated with a significant increase in fasting serum gastrin levels. Moreover the gastrin increase levels gastric also elevated the PGE<sub>2</sub> in the gastric mucosa and maintains mucosal

integrity. Consequently, gastrin may indirectly stimulate gastric PG synthesis in mucosa epithelium (Komori et al., 2002).

Our results showed that PAs extract reduced the ulcerative lesion index on ethanol induced-gastric ulcers and increase the gastrin plasma levels. Probably this increase of gastrin levels promote consequently an increase of PGE<sub>2</sub> levels and a cytoprotection.

We also evaluated the pharmacological activity of the PAs extract in the acetic acid-induced gastric ulcer (Chronic gastric ulcer) with the administration of this alkaloidal extract (12.5 mg/Kg, p.o.) for 14 days and measured the possible increase of the expression of the EGF by mRNA

Chronic gastric ulcer is a deep necrotic lesion involving the entire mucosal depth and penetrating through the muscularis mucosae. Ulcer healing is a dynamic process of filling mucosal defects with proliferating and migrating epithelial cells and connective tissue resulting in reconstruction of the mucosal architecture (Tarnawski et al. 1995).

Several works focused the antiulcerogenic process related to the healing process and the participation of the protein of epidermal growth factor (EGF). EGF stimulate cell proliferation and is involved in regulation of growth in the gastrointestinal tract under normal physiological conditions. It is known that EGF stimulates DNA synthesis in the gastroduodenal mucosa of rats and accelerates ulcer healing (Mutsaers et al. 1997). EGF act on its target cells after binding to specific membrane receptors that have been identified in the epithelial cells of the mucosa of the stomach and small intestine (Murphy et al. 1998)

The expression of EGF mRNA was detected using reverse transcriptase polymerase chain reaction in gastric mucosa of the ulcerated group that received the PAs fraction (12.5 mg/kg, p.o.). RT-PCR analysis clearly demonstrated the more evident expression of mRNA after the treatment with PAs extract when compared to the respective control group.

Several investigators have reported that the increase of the gastrin levels also stimulated the production of one of the members of growth factors family, the epidermal growth factor (EGF) (Tarnawski et al. 2001). We concluded that PAs increase the levels of gastrin, consequently promoted an elevation of the expression of mRNA of EGF in the gastric mucosa and demonstrated a quality of the cicatrization in the gastric mucosa.

In the evaluation of the histology, the damage areas of the saline treatment group included exfoliation and necrosis of superficial cells, generalized blood cell infiltration and

evident signs of reperfusion in comparison with non-ulcerated gastric mucosa and lansoprazole treatment. The treatment with PAs demonstrated a reduction of this aggressive factor in the gastric mucosa of these animals.

Our present study demonstrated that PAs extract showed an antiulcerogenic activity on acute and chronic induced gastric ulcers. PAs elevated the gastrin serum plasma levels and probably increase the PGE<sub>2</sub> in the gastric mucosa, to provide a cytoprotection. Furthermore, the increases of gastrin levels after administration of PAs activates EGF expression on ulcerated gastric mucosa and contribute to the quality of cicatrisation process. The histological examinations showed the minor aggression on the mucosal tissue in the animals that received PAs treatment.

We concluded that PAs extract provides excellent preventive and curative effects on acute and chronic induced gastric ulcers. They probably related to be a modulation of the levels of the gastrin hormone and, consequently, increase the expression of the PGE<sub>2</sub> and EGF protein that induce a increase of the quality of this cicatrisation process.

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### **2.2.6- Resumo do artigo II**

Tendo em vista que os PAs apresentam atividade antiulcerogênica gástrica e duodenal na dose de 12.5 mg/Kg, este trabalho tem como objetivo avaliar se esta atividade antiulcerogênica é evidenciada não apenas quando administrada agudamente, mas também com o uso crônico desta fração alcaloidal. Além disso, este artigo objetiva avaliar a participação destes PAs na capacidade em aumentar a síntese pelo RNAm da proteína do fator de crescimento epidermal (EGF), fator este que garante não apenas tempo mais hábil de cicatrização das lesões ulcerogênicas, como também caráter qualitativo neste processo de regeneração e também dos níveis de secreção do hormônio gastrina, extremamente relacionado a este processo regenerativo.

Os resultados crônicos relatam intensa redução dos níveis de lesões ulcerogênicas bem como aumento da expressão do EGF em comparação aos demais grupos tratados. Os níveis plasmáticos de gastrina também apresentam-se aumentados, sendo isto um fator chave para o estímulo do aumento da expressão do EGF.

Estes resultados em conjunto vêm a corroborar com os resultados obtidos anteriormente e demonstrando que os PAs podem ser fontes alternativas futuras para o tratamento das lesões ulcerogênicas.



### **2.3- Artigo III: em processo final para envio**

#### **2.3.1- Objetivos do artigo III:**

- A) Avaliar a atividade antiulcerogênica de PAs de *Senecio brasiliensis* no modelo de indução de lesões gástricas por Isquemia-reperfusão (IR) em ratos
- B) Avaliar a provável atividade antioxidante dos PAs após indução de IR em ratos através da quantificação da enzima mieloperoxidase (MPO)
- C) Avaliar as características histológicas da mucosa gástrica dos animais tratados com PAs após IR em comparação aos grupos controle

#### **2.3.2- Título do Artigo III:**

Gastroprotection induced by pyrrolizidine alkaloids from *Senecio brasiliensis* in oxidant stress induced by Ischemia-Reperfusion in rats

#### **2.3.3- Revista a ser submetido:**

A ser definida

### **2.3.4-- Artigo III**

**Gastroprotection induced by pyrrolizidine alkaloids obtained from *Senecio brasiliensis* in oxidant stress by ischemia-reperfusion in rats**

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### ***Abstract***

The generation of oxygen-derived free radicals has been suggested to be significantly responsible for ischemia-reperfusion injury in gastrointestinal tissues. The changes in the activities of related enzymes such as myeloperoxidase (MPO), a marker enzyme of is related to this pathologic process. We investigated the antiulcerogenic activity of pyrrolizidine alkaloids (PAs, an alkaloidal fraction obtained from ethanolic extract of *Senecio brasiliensis* in the ischemia-reperfusion induced gastric ulcer and measured the levels of MPO on these animals. The results showed a significantly antiulcerogenic activity ( $p < 0.0001$ ) of PAs at three doses (6.25, 12.5 and 50  $\text{mg}\cdot\text{kg}^{-1}$ , p.o.). However, only PAs 12.5 and 50  $\text{mg}/\text{Kg}^{-1}$  showed a significative reduction of leucocytes migration by after measured the MPO levels, when compared to the respective control values. Moreover, the histological examinations showed a reduction of exfoliation of superficial cells, hemorrhages and blood cell infiltration. We concluded that the PAs fraction presented an important antiulcerogenic activity related to the antioxidant activity.

## ***Introduction***

Gastric, duodenal or intestinal ulcers are deep necrotic lesions penetrating through the entire mucosal thickness and the muscularis mucosae (1). The etiology of this pathology is influenced by various aggressive and defensive factors such as acid-pepsin secretion, parietal cell, mucosal barrier, mucous secretion, blood flow, cellular regeneration, and endogenous protective agents such as prostaglandins and epidermal growth factors (2).

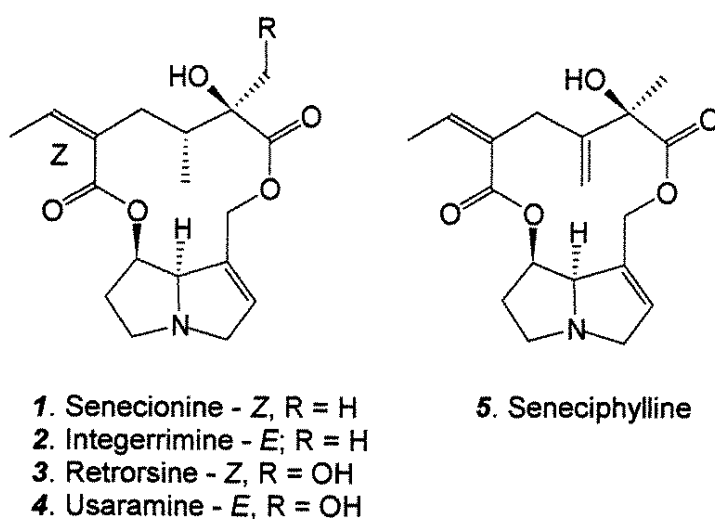
In the last years, several works related that the oxygen-derived free radicals have been postulated to play an important role in the pathogenesis of acute gastric mucosal injuries such as stress, ethanol, anti-inflammatory drug and ischemia-reperfusion-induced gastric mucosal injuries in rats (3).

It is known that the ischemia-reperfusion (IR) changes may cause acid and pepsin to attack the gastric mucosa, producing erosion or an ulcer (4). Studies of IR injury in the intestine and stomach implicate both xanthine-oxidase (XO)-derived oxidants and neutrophils in the pathogenesis of this disorder. XO-derived oxidants, mainly the superoxide anion, seem to elicit the production of inflammatory mediators which attract and activate polymorphonuclear neutrophils (PMN) into postischemic injury. It is known that circulating PMN cause gastric mucosal injury by generating oxygen free radicals. Myeloperoxidase (MPO), which is an important marker enzyme of these PMN (5).

Actually, numerous natural substances have been suggested to act as antioxidants. Antioxidants act as radical scavengers, inhibit lipid peroxidation and other free radical-mediated processes, and therefore they protect the human body from several diseases attributed to the reactions of radicals (2).

*Senecio brasiliensis* (Asteraceae), is a native species from South America found in south and southeast Brazil. The leaves and inflorescences of *Senecio brasiliensis* are utilized by traditional medicine for the treatment of stomach pain (6). Popularly, this plant is known as "Flor das Almas", "Tasneirinha", "Margaridinha" or "Maria-Mole", (7, 8).

Phytochemical studies carried on the *Senecio brasiliensis* plant revealed the presence of the pyrrolizidine alkaloids integerrimine (2), retrorsine (3); as well as their respective geometrical isomers, senecionine (1), usaramine (4) and seneciophylline (5) (Figure 1) (9, 10). Recently, we reported the antiulcerogenic activity of these pyrrolizidine alkaloids (PAs), in different ulcerogenic models in rats and mice (11).



**Figure 1. Chemical structures of pyrrolizidine alkaloids isolated from *Senecio Braziliensis* inflorescences.**

The present study was undertaken to explore the additional mechanisms responsible for PAs-induced gastroprotection in IR injury gastric. Therefore, the enzymatic activities related with oxidative stress such as myeloperoxidase (MPO), were studied in an experimental animal model using reefered rats. Moreover we evaluated the histological characteristics of the gastric mucosal after this experimental procedure.

### **Material and methods**

#### ***Animal groups and drug preparation***

The experiment were performed on Male and female Wistar rats (6-8 each group), 180-200 g, obtained from Animal Service, University of Seville. The animals were placed singled with wire-net floors in a controlled room of temperature (22-24 °C), humidity 70-75%, 12h light/dark cycle and were fed a normal laboratory diet. Rats were deprived of food for 24 h before experimentation but allowed free access to tap water throughout. In the present study both male and female rats were mixed, because the previous experiments

undertaken in the laboratory of University of Seville, the sex had not influence in the incidence and severity of IR induced gastric injury. The experimental protocol was approved by the Animal Use and Care Committee of University of Seville and was conducted in accordance with the recommendations of the European Union. PAs from *Senecio brasiliensis* was freshly dissolved in 0.9% NaCl and was administered by oral route 30 min before experimentation. Control rats received the vehicle only in comparable volume also by enteral route. The animals were randomly divided into five groups: control (non-ulcerated), ischemia-reperfusion (IR) ulcerated control and pyrrolizidine alkaloids (50, 12.5 and 6.25 mg Kg<sup>-1</sup> B.W.) groups.

### **Pharmacological Assay**

#### ***Gastric injury***

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg Kg<sup>-1</sup> B.W. The left side of the abdomen was shaved and a 3 cm incision was made from the midline to below the ribcage using a diathermy. The celiac artery was dissected free of excess fat and clamped for 30 min approximately 0.5 cm from its origin from the aorta, using an atraumatic microvascular clamp. Reoxygenation was allowed by removal of the clamp for 60 min (12). At the end of the experimental period, the animals were killed by exsanguination via the abdominal aorta. The stomach of each rat was removed and opened along the great curvature and any lesions were examined macroscopically; the number and area of gastric lesions were determined using a planimetry (Morphomat, Carl Zeiss, Berlin, Germany) by one investigator who was unaware of the treatment given. The length and width of the ulcers were measured on a cold stand and the sum of damaged areas was calculated. Results were expressed as percentage of damage. Then, the mucosa was scrapped off by means of two glass slides on ice, weighed and frozen at -70°C until biochemical determinations. In 6 rats of each group, samples of macroscopically normal and ulcerated stomachs were processed by routine methods for subsequent histological evaluation.

### ***Assessment of leukocyte involvement***

Neutrophil infiltration in vivo has previously been assessed by measuring granulocyte specific enzymes, such as myeloperoxidase (MPO), in tissue (13, 14).

### ***Myeloperoxidase assay***

MPO activity was assayed spectrophotometrically using a minor modification of the method that utilizes 3,3',5,5'-tetramethylbenzidine (TMB) as substrate (13). 20  $\mu$ l of homogenate was added to a 0.2 ml reaction volume containing 80 mM PBS (pH 5.4), 0.5% HETAB and 1.6 mM TMB. The mixture was incubated at 37 °C for 5 min and the reaction started by the addition of 0.3 mM H<sub>2</sub>O<sub>2</sub>. Each tube containing the complete reaction mixture was terminated by the sequential addition of catalase (20  $\mu$ g/ml) and 2 ml of 0.2 M sodium acetate (pH 3.0). The changes in absorbance at 655 nm were measured with a spectrophotometer (Model Perkin-Elmer Lambda 3). One unit of MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 Unit/min at 37 °C in the final reaction volume containing the acetate.

### ***Protein assay***

Proteins were determined using the Bradford procedure, with albumin as standard (15).

### **Statistical analysis**

The results are presented as mean  $\pm$  SD. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnet's test, with the level significance set at  $p < 0.05$ .

### **Discussion**

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (16). Natural compounds can be lead compounds, allowing the design and rational planning of new



drugs, biomimetic synthesis development and the discovery of new therapeutic properties not yet attributed to known compounds (17).

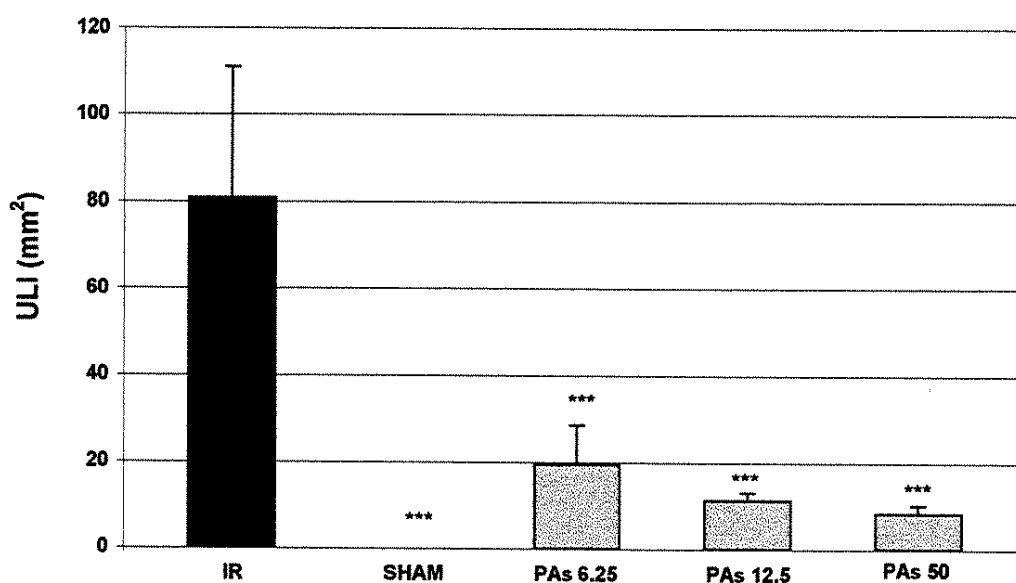
In the last years, eminent attention has been showed to the drugs as antioxidant preventive and curative activities. Spices and herbs are recognized as sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the chemoprevention of diseases resulting from lipid peroxidation (2).

Experimental evidences suggest that the generation of oxygen-derived free radicals is significantly responsible for IR injury in gastrointestinal tissues. These oxygen free radicals can attack and initiate a free radical chain reaction known as lipid peroxidation (18).

In the present study, we evaluated the pharmacological activity of the PAs fraction extract obtained from *Senecio brasiliensis* on the IR induced gastric ulcers and measured the levels of infiltration of the polymorphonuclear leukocytes (neutrophils) (PMN) after this treatment by the myeloperoxidase (MPO) activity.

We can observed that the administration of PAs by oral route at doses of 6.25, 12.5 and 50 mg.kg<sup>-1</sup>, markedly reduced the ulcerative lesion index in rats submitted to the IR by 76.0, 86.0 and 90.0%, respectively (Figure 2) when compared to the respective control values.

**Figure 2:** Ulcer index (mm<sup>2</sup>) after administration of PAs fraction extract on ischemia-reperfusion gastric injury.



Data are presented as the mean  $\pm$  SD (n =6-8). ANOVA following F values:  $F_{(4,29)}=34.834$  ( $P<0.05$ ). Dunnett's test \*\*\* $P<0.0001$

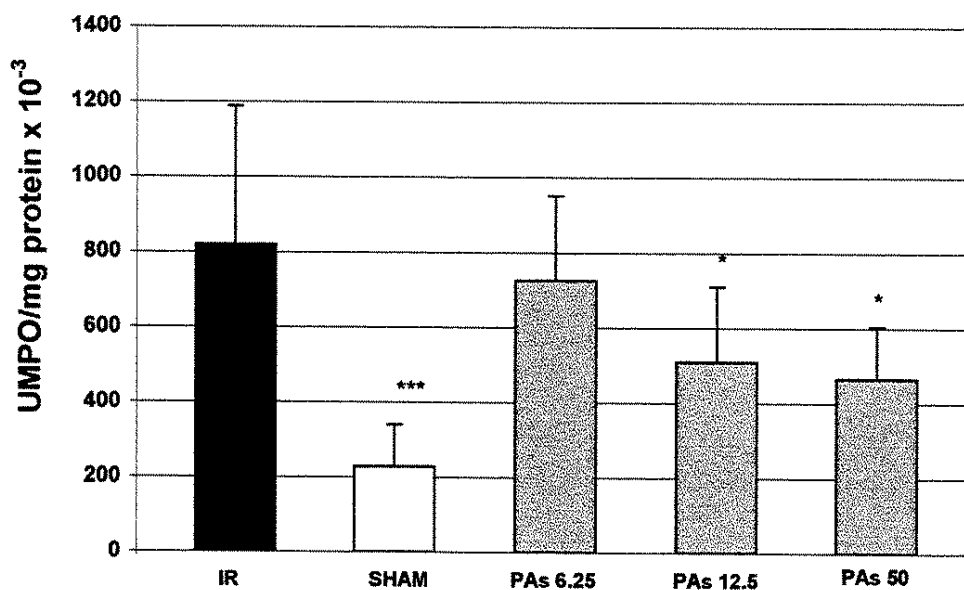
The gastric acid secretion plays an important role in the progression of post IR erosions to gastric ulcers and drugs that suppress this secretion such as histamine H<sub>2</sub> receptor antagonists or proton pump inhibitors can be useful in acceleration of healing of these lesions (19). These findings are consistent with the previous results already obtained with this fraction extract, when the PAs protects the gastric mucosa from damage caused by a variety agents such as HCl-ethanol, indomethacin-bethanecol, stress and reduced the gastric acid secretion in the pylorus ligation (11).

Several works related the biochemical mechanisms involved on the pathophysiology of the IR. These radicals to be produced include the XO system, that on reperfusion release of superoxide radicals and H<sub>2</sub>O<sub>2</sub>. These oxygen radicals may then be converted to the highly cytotoxic hydroxyl radical by the iron-catalyzed Haber-Weiss reaction. This initiates the process of lipid peroxidation and release of substances that recruit and activate PMN (5).

Our results show the presence of the leukocyte reaction as indicated by the values of UMPO activity. PAs fraction extract showed the reduction of the levels of the MPO in the three doses utilized (Figure 3), but only doses of 12.5 and 50 mg.kg<sup>-1</sup> demonstrated significant activity (p<0.05).

PMN have been implicated have been implicated in the pathogenesis of many forms of gastrointestinal injury induced by a variety factors, including the IR (20). Neutrophils emigrate into the interstitial space promoted by chemotactic agents and, thereafter, release oxygen-derived free radicals and proteases, resulting in neutrophil-dependent inflammatory tissue injury (21). Therefore, the PAs fraction extract can be too promoted the reduction of the IR lesion by the reduction of the PMN levels and consequently decrease of the oxygen-derivated free radicals.

**Figure 3:** UMPO/mg protein x 10<sup>-3</sup> after administration of PAs fraction extract on ischemia-reperfusion gastric injury.



Data are presented as the mean ± SD (n =6-8). ANOVA following F values:

F<sub>(4,29)</sub>=7.496 (P<0.05). Dunnett's test \*P<0.05; \*\*\*P<0.0001

We also evaluated the histological characteristics of the gastric tissue after ischemia-reperfusion procedure. Histologically, the damage areas of the saline group included edema, hemorrhages, exfoliation and necrosis of superficial cells and generalized blood cell infiltration (Figure 4) in comparison with non-ulcerated gastric mucosa (Figure 5). Treatment with 50 mg/Kg<sup>-1</sup> of PAs clearly diminished the damage (Figure 6).

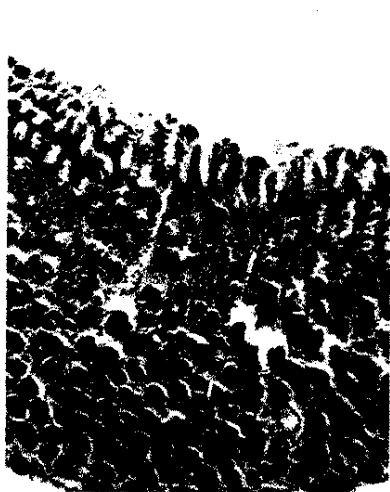


Figure 5



Figure 4

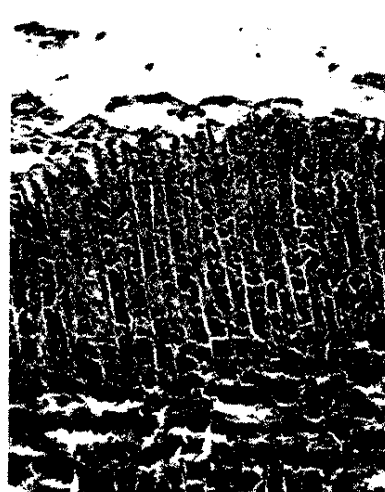


Figure 6

In this study, we concluded that the PAs obtained from *Senecio brasiliensis* is a potential natural compound with the antiulcerogenic activity. The PAs fraction extract significantly prevent the formation of gastric lesions induced by IR in rats, and this activity could be due, in part, to their capacity of the reduction of gastric acid secretion and/or by antioxidant activity, because the your capacity for scavenging of oxygen free radicals.

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### **2.3.5- Resumo do artigo III**

A indução de lesões gástrica em ratos submetida à Isquemia-reperusão (IR) é um dos mais novos modelos experimentais utilizados visando avaliar a provável atividade antiulcerogênica via mecanismos antioxidantes. A fração alcaloidal de PAs demonstra atividade antiulcerogênica significativa após IR em ratos, demonstrando com isso grandes chances do envolvimento da atividade antioxidante deste grupo de compostos farmacologicamente ativos.

Após quantificação dos níveis da enzima relacionada ao processo inflamatório-oxidativo, a mieloperoxidase (MPO), os resultados com tratamento com PAs demonstram significativa queda nos níveis desta enzima, fator este que comprova a atividade antioxidante dos PAs dentre os mecanismos antiulcerogênicos destes compostos.

Este resultado torna-se extremamente relevante, uma vez que atualmente vem crescendo a vertente da pesquisa científica na área na busca de substâncias com potencial atividade antioxidante. Com isto, torna-se ainda mais evidente de que os PAs são fontes de novas perspectivas na terapêutica de novos fármacos com potencial atividade antiulcerogênica.



### **3. CONCLUSÕES**

Os resultados obtidos confirmam a atividade antiulcerogênica gástrica e duodenal dos PAs tanto administrados aguda e cronicamente. A dose utilizada (12.5 mg/Kg) é uma dose considerada viável, uma vez que apresenta certa margem de segurança quando comparada à dose letal 50 (234.4 mg/Kg). A atividade citoprotetora, a modulação da secreção dos hormônios gastrointestinais gastrina e somatostatina e o aumento da expressão do fator de crescimento epidermal (EGF) são os mecanismos farmacológicos relacionados à atividade antiulcerogênica demonstrada pelos PAs. Além disso, mecanismos antioxidantes são também evidenciados com a redução das lesões induzidas por isquemia-reperfusão. A redução quantitativa dos níveis da enzima mieloperoxidase (MPO) também é um resultado que fortifica a atividade antioxidante desta fração alcaloidal nas lesões ulcerogênicas.

O trabalho demonstra com isso que os PAs, apesar de serem considerados substâncias hepatotóxicas, podem também apresentar atividades benéficas na patologia da úlcera gástrica. Isto torna claro a necessidade de novos estudos tanto do ponto de vista químico quanto farmacológico, uma vez que os PAs podem vir a ser num futuro próximo uma das novas vias alternativas para o tratamento de patologias do trato gastrointestinal, sobretudo as lesões ulcerogênicas.

## 4. SUMMARY

*Senecio brasiliensis* is a native plant from South América and is found mainly in Brazil. Popularly *Senecio* is knowed as Margaridinha, Flor-das-Almas, Tasneirinha and is very utilized by folk medicine to the treatment of gastrointestinal diseases. Phytochemical analysis showed the presence of Pyrrolizidine Alkaloids (PAs) from *Senecio brasiliensis*. This study have a objective the obtained of PAs extract and evaluated the antiulcerogenic activity of the PAs on standard rodent models. Furthermore this study also evaluated the probably pharmacological mechanism(s) of the PAs. The results showed the antiulcerogenic activity on gastric and duodenal induced ulcerogenic lesions. The pharmacological mechanisms showed that PAs can be a quantitative and probably qualitative antiulcerogenic activity. PAs, therefore is a probably therapy alternative in the future. News assays are necessary to determinate the efficacy and safety of this drug.

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## **6. APÊNDICE**

### **6.1- Certificado estágio exterior (PDDE)**

- **Instituição:** Universidade de Sevilha, Faculdade de Farmácia, Departamento de Farmacologia
- **Convênio:** PDDE (CAPES)
- **Objetivos:** aprendizado da metodologia de indução de úlcera gástrica por isquemia-reperfusão em ratos e análise dos ensaios bioquímicos posteriores.
- **Período:** Setembro 2003 a Janeiro 2004.
- **Orientadora:** Prof. Dra. Catalina Alarcón de la Lastra



DEPARTAMENTO DE FARMACOLOGIA  
FACULTAD DE FARMACIA  
UNIVERSIDAD DE SEVILLA

Dr. D. Luis Bravo Díaz Catedrático y Director del Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla,

INFORMA: que Walber Toma Becario del Departamento de Fisiología y Biofísica del Instituto de Biología de la Ciudad Universitaria Zeferino Vaz de Campinas, Sao Paulo, Brasil ha realizado su estancia con dedicación exclusiva y con resultados muy positivos en los Laboratorios del Departamento durante los meses de Septiembre a Diciembre ambos inclusive del presente año académico.

Y para que así conste y a petición del interesado, firmo el presente informe, a diez y siete de diciembre de dos mil dos.

Fdo.: Luis Bravo Díaz

## 6.2. Participação em Congressos

- **XVIII Reunião Anual da Federação de Sociedades de Biologia Experimental – FESBE**
- Realizada na cidade de Pinhais-PR, 27-30 Agosto 2003.
- Trabalho apresentado sob a forma de comunicação oral
- **Título:** Atividade antiulcerogênica de Alkalóides Pirrolizidínicos obtidos a partir de *Senecio brasiliensis* em stress oxidativo gerado por isquemia-reperfusão em ratos

# FESBE 2003

Certificamos que

o trabalho Atividade antiulcerogênica de alcalóides pirrolizidínicos obtidos a partir de *senecio brasiliensis* em stress oxidativo gerado por isquemia-reperfusão em ratos de autoria de Toma, W. ; Alarcón de la Lastra, C. ; Trigo, J. R. ; Souza Brito, A. R. M. foi apresentado sob a forma de comunicação oral no Módulo Temático Fisiofarmacologia das doenças gastrointestinais na

XVIII Reunião Anual da  
Federação de Sociedades de Biologia Experimental-FeSBE,  
realizada na cidade de Pinhais - Paraná, de 27 a 30 de agosto de 2003.



Comissão Organizadora

### 6.3- Publicações Científicas

- **Revista:** Phytomedicine (*In Press*) (2003)
- **Título:** Evaluation of the antiulcerogenic activity of extracts obtained from *Mammea americana*. A new potent pharmacological alternative.
  
- **Revista:** Journal of Ethnopharmacology Vol 85(1): 19-23 (2003)
- Título:** Evaluation of the analgesic and antiedematogenic activities of *Quassia amara* bark extract
  
- • **Revista:** Biological Pharmaceutical Bulletin Vol. 25(4): 452-456 (2002)
- **Título:** Natural trans-Crotonin: The Antiulcerogenic Effect of Another Diterpene Isolated from the Bark of *Croton cajucara* Benth.
  
- • **Revista:** Phytomedicine Vol. 8(2), 94-100, March 2001.
- **Título:** Gastroprotective effect of Aparisthman, a diterpene isolated from *Aparisthimium cordatum*, on experimental gastric ulcer models in rats and mice.
  
- • **Revista:** Biological Pharmaceutical Bulletin, Vol. 23(12), 1465-1469 (2000).
- - **Título:** Evaluation of the Gastroprotective Activity of Cordatin, a Diterpene Isolated from *Aparisthimium cordatum* (Euphorbiaceae).