

Antiulcerogenic activity of a crude hydroalcoholic extract and coumarin isolated from *Mikania laevigata* Schultz Bip

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Abstract

The leaves of *Mikania* (Asteraceae) species are used in folk medicine as antispasmodic, antiulcerogenic and antirheumatic agents. Phytochemical screening of the crude hydroalcoholic 70% extract (CHE) of *Mikania laevigata* Shultz Bip. revealed coumarins, terpenes and organic acids. Antiulcerogenic activity of CHE was evaluated, employing different experimental models in rats, to discern the pharmacological mechanism of action. Both the antisecretory and the cytoprotection hypothesis were evaluated. The crude hydroalcoholic extract (1000 mg/kg body wt., vo) decreased the ulcerative lesion index produced by indomethacin, ethanol, stress and reserpine in rats by 85%, 93%, 82% and 50%, respectively. In the pyloric ligation model, a decrease of hydrogenionic concentration (53%) was observed, suggesting that the pharmacological mechanism has a relationship to antisecretory activity. The antisecretory mechanisms of CHE and the coumarin isolated from *M. laevigata* were confirmed by acid hypersecretion induced by histamine, pentagastrin and bethanechol. Duodenal administration of CHE (1000 mg/kg body wt.) and coumarin (100 mg/kg body wt.) inhibited only the gastric acid secretion produced by bethanechol. These results suggest that both CHE and coumarin may influence the secretion control mediated by the parasympathetic system.

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Keywords: *Mikania laevigata*; Antiulcerogenic activity; Antisecretory activity; Coumarin; 2H-1-benzopyran-2-one; Medicinal plant

Introduction

Available evidence indicates that gastric acid secretion disorders, as well as gastric mucosal integrity alterations, may contribute simultaneously to the multi-

factorial pathogenesis of peptic ulcers. Concomitant *Helicobacter pylori* infection may contribute to the pathology's intensity (Barocelli et al., 1997).

The current medicinal treatment of peptic ulcer is generally based on the inhibition of gastric acid secretion by H₂-blockers, omeprazole and antimuscarinics, as well as on the acid-independent therapy provided by sucralfate and bismuth. In the case of *H. pylori* infection, antibiotics are used. Obviously, drugs endowed with antisecretory activity coupled with gastroprotective effects could represent a

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promising approach for successful treatment of peptic gastric ulcer because of potential complementary effects of therapeutic modalities acting via different mechanisms (Barocelli et al., 1997).

Medicinal plants have an important therapeutic role in the treatment of many human illnesses (Pezzuto, 1997) and have long been a source of medicines. Nowadays almost 25% of the active components of currently prescribed medicines were first identified in higher plants. Six of the top 20 pharmaceutical drugs sold in 1996 were natural products and more than 50% of this top 20 were linked directly to natural product research (Balandrin et al., 1996).

Most of the population in developing countries still relies on traditional medicine practitioners and local medicinal plants for primary health care. In Brazil, the use of crude plants extracts, infusions or plasters is a widespread practice in the treatment of pathologies (Barros et al., 1970).

As an alternative to other approaches (Garcia et al., 1978; Rimbau et al., 1999), scientific research on plants used in traditional medicine is receiving growing interest as a way of identifying new agents.

Mikania (Asteraceae) species are found throughout tropical regions of Africa, Asia and South America (Argentina, Paraguay and Uruguay). In Brazil, they are found mainly in the South and Southwest of the country. Commonly known as “guaco”, the plant grows as a timbered shrub with a branched cylindrical stem (Castro et al., 1986).

The leaves of *Mikania* species are used in folk medicine as antispasmodic, antiulcerogenic and anti-rheumatic agents (Gupta, 1994; Pereira et al., 1994; Mosaddik and Alam, 2000). Certainly, *Mikania* species are one of the most-used medicinal plants in Brazil. Folk medicine and public health centers offer tea, tinctures and a variety of extracts from this species to treat asthma, respiratory problems and other infections. Many *Mikania* species are cultivated at the CPQBA/UNICAMP experimental field. Since 1995, *M. laevigata* has been under research there, to evaluate the agronomical parameters of harvest under sun and shade conditions.

Owing to wide use in Brazil and in other parts of South America (Castro et al., 1986), our group investigated the antiulcerogenic activity and the probable pharmacological mechanism of a crude hydroalcoholic 70% extract (CHE), as well as the isolated coumarin, obtained from *M. laevigata* leaves.

Materials and methods

Animals

Male Wistar rats, weighing 200–300 g each and fasted for 24 h before each experiment, were offered free access

to water. These animals were maintained under a standard light cycle (12 h light, 12 dark) and temperature (20 °C), for at least 7 days before the experiments. Animal welfare guidelines were observed during maintenance period and experimentation.

Drugs and reagents

Indomethacin, cimetidine, carbenoxolone, atropine, reserpine, histamine, pentagastrin and bethanecol were purchased from Sigma (St. Louis, MD, USA). All other reagents used were of analytical grade.

Plant material and extract preparation

The aerial parts of *M. laevigata* were collected from the experimental field of CPQBA/UNICAMP. A voucher specimen has been deposited at the IB/UNICAMP Botany Department under registration number UEC-102.046.

This material was allowed to dry under air circulation (40 °C) and ground for use. The resulting powder (200 g) was submitted to dynamic maceration with ethanol 70% (3 × 1 l) for 4 h. This procedure was repeated three times with the same powder. After filtration, the residue was discarded and the solvent evaporated at 40 °C in vacuo and freeze-dried, yielding a green powder (40 g), designated as the CHE, which was stocked in a dry chamber at –20 °C. The extraction yield was 20%.

Coumarin 1 isolation

The CHE (30 g) was purified by column chromatography on Silica gel (Merck 7734)(4 × 80 cm), using gradients of hexane/ethyl acetate (95:5), Rf: 0.58 between 1100 and 1400 ml. Fractions (100 ml): were monitored by thin layer chromatography (aluminum sheets Merck 1.05554), detection reagent anisaldehyde, eluent: hexane/ethyl acetate 35%, mp 69–70°. The physical and spectral data (ms, ¹H-NMR, ¹³C-NMR) of Compound 1 were consistent with literature reports.

Gas chromatography with mass spectroscopy (GC/MS) analysis was carried out using an HP-5890/5970 system equipped with a JandW Scientific DB-5 fused capillary column (25 m × 0.2 mm × 0.33 m). Temperature program 110 °C (2 °C/min.)–300 °C (10 min). Injector equal to 250 °C and detector temperature equal to 280 °C. Helium was the carrier gas (0.7 bar, 1 ml/min). The MS were taken at 70 eV. Scanning speed was 0.84 scans/s from 40 to 550. Sample volume was 1 µl. Split 1:40

Indomethacin-induced ulcer

The animals were divided into at least three groups, according to the treatment employed (saline 10 ml/kg body wt.; cimetidine 100 mg/kg body wt.; CHE 1000 mg/kg body wt.).

After 30 min oral treatment, indomethacin (30 mg/kg body wt.) was administered subcutaneously to all animal groups, as described by Morimoto et al. (1991).

After 4 h, the animals were sacrificed by cervical displacement and their stomach were removed, and opened along the greater curvature. The ulcerative lesion index (ULI) of each animal was calculated by adding the following values, according to the method of Gamberini et al. (1991).

Loss of normal morphology	1 point
Discoloration of mucosa	1 point
Mucosal edema	1 point
Hemorrhages	1 point
Petechial points (until 9)	2 points
Petechial points (> 10)	3 points
Ulcers up to 1 mm	$n \times 2$ points*
Ulcers > 1 mm	$n \times 3$ points*
Perforated ulcers	$n \times 4$ points*

*Number of ulcers found.

Absolute ethanol-induced ulcer

The animals were divided into groups according to the treatment employed (saline 10 ml/kg body wt.; carbenoxolone 200 mg/kg body wt.; CHE 700 mg/kg body wt.; or 1000 mg/kg body wt.).

After 30 min oral or subcutaneous treatment, each animal received 1 ml of absolute ethanol orally, according to the method of Robert (1979). After 1 h, the animals were sacrificed by cervical displacement and their stomach were removed and opened along the greater curvature. The ulcerative lesion index was determined as above (Gamberini et al., 1991).

Hypothermic restraint stress-induced ulcers

The animals were divided in three groups according to the treatment employed (saline 10 ml/kg body wt.; cimetidine 100 mg/kg body wt.; CHE 1000 mg/kg body wt.). After 30 min oral treatment, the animals were placed in a refrigerated room at 4 °C for a period of 2 h. After this period, the animals were sacrificed by cervical displacement and their stomachs were removed and opened along the greater curvature (Levine, 1971). The ulcerative lesion index of each animal was calculated as above (Gamberini et al., 1991).

Reserpine-induced ulcer

The animals were divided into three groups according to the treatment employed (saline 10 ml/kg body wt.; atropine 10 mg/kg body wt.; CHE 1000 mg/kg body wt.). After 30 min oral treatment, reserpine (10 mg/kg body wt.) was administered intraperitoneally to all animals groups according to the method of Gupta et al. (1974). After 20 h, the animals were sacrificed by cervical displacement and their stomach were removed and opened along the greater curvature. The ulcerative lesion index was determined as above (Gamberini et al., 1991).

Pyloric ligation

The animals were divided into groups according to the treatment employed (saline, cimetidine, CHE and coumarin 1). The animals' abdomens were incised under ether anesthesia and the pylorus was ligated. Immediately after this procedure, saline solution (2.5 ml/kg), CHE (1000 mg/kg body wt.), cimetidine (100 mg/kg body wt.) or coumarin 1 (100 mg/kg body wt.) were administered intraduodenally to the respective animal groups. The abdominal wall was sutured and after 4 h, the animals were sacrificed by cervical displacement. Their stomachs were removed after cardial ligation. The gastric content was centrifuged and the gastric juice volume was measured. The hydrogenionic concentration was determined by titration of gastric juice with NaOH solution (0.1 N), according to the method of Shay et al. (1945).

The model described previously was also used to establish the effect of CHE and coumarin 1 on gastric secretion induced by histamine administration (25 mg/kg body wt., sc), pentagastrin (4 µg/kg, ev) and bethanechol (1.5 mg/kg body wt., sc). These secretagogues were administered after 1 h of pyloric ligation (Fig. 1).

Statistical analysis

The results were expressed as mean ± standard deviation and the individual data were submitted to one-way variance analysis (ANOVA) with critical range at $p < 0.05$, and after to Duncan's test with the same critical range.

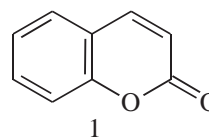


Fig. 1. Formula of coumarin 1.

Results and discussion

Dry ground *M. laevigata* leaves were macerated with 70% ethanol, yielding the CHE at 20%. The presence of a coumarin, terpenes, and organic acids was detected in CHE via phytochemical screening.

The indomethacin-induced ulcer model was employed for screening antiulcerogenic activity, because the model shows cytoprotection and gastric acid secretion (Morimoto et al., 1991; Katori and Majima, 1997). Four different doses (250, 500, 1000 and 2000 mg/kg body wt.) of CHE, administered orally, inhibited the ulcerative lesion index by 49, 60, 85 and 92%, respectively. An ED₅₀ value of 564.0 mg/kg body wt. was determined by linear regression.

In the ethanol-induced ulcer model, used to screen drugs for cytoprotection (Robert 1979), CHE inhibited the ulcerative lesion index by 93% when administered orally.

Although *M. laevigata* is used in folk medicine for respiratory infections, these results demonstrated better inhibition of the ulcerative lesions than the *Maytenus ilicifolium* extracts known as antiulcerogenic agents (Souza-Formigoni et al., 1991; Queiroga et al., 2000). Our interest was therefore aroused in identifying and determining the pharmacological mechanism of the active compounds.

The hypothesis that antiulcerogenic activity of CHE may be caused by a local and nonspecific mechanism called adaptive cytoprotection was investigated. In order to verify this possibility, subcutaneous administration of CHE was evaluated on absolute ethanol-induced ulcer model. Cytoprotection may occur as result of the capacity that some compounds have to induce prostaglandin production, fundamental for mucus protection,

because they stimulate mucus and bicarbonate synthesis (Robert et al., 1983). A forty-percent inhibition of the ulceration lesion index was observed, reinforcing the hypothesis that antiulcerogenic substances present in CHE act through a specific systemic mechanism (Table 1).

High reserpine doses produce an intense generalized discharge of sympathetic nervous system mediators, inducing ulcers within 24 h (Gupta et al., 1974). Under that chemical experimental stress model, CHE inhibited the ulcerative lesions by 50%. Under the experimental stress model, induced by immobilization at low temperatures, the crude ethanol extract inhibited ulcerative lesions by 82% (Table 1).

Stress, chronic use of anti-inflammatory drugs and alcoholic beverages are some of the main causes of gastric ulcers (Barocelli et al., 1997). Since CHE produced significant inhibition of the ulcer lesions under experimental models that simulate those conditions, priority was given to the determination of the pharmacological mechanism.

The pyloric ligation model described by Shay et al., and the use of endogenous substances that stimulate gastric acid secretion, were chosen (Schubert, 1994). Parameters such as volume and hydrogenionic concentration were evaluated in treated animals with CHE at a dose of 1000 mg/kg body wt. and coumarin 1 at a dose of 100 mg/kg body wt.

The CHE (1000 mg/kg body wt.) and coumarin 1 (100 mg/kg body wt.) in the pyloric ligation model, without administration of the secretagogue, reduced the volume by 76% and 51%, with hydrogenionic concentration reduction of 53% and 47%, respectively (Table 2).

Table 1. Effect of a CHE obtained from *M. laevigata* Schultz. Bip., in absolute ethanol-, hypothermic restraint-stress and reserpine-induced ulcer models

Model	Treatment	n	Route	Dose (mg/kg body wt.)	ULI (mean ± s.e.m.)	Inhibition (%)
Ethanol ^a	CHE	7	Vo	1000	11.0 ± 4.2*	93.0
	Carbenoxolone	7	Vo	200	22.9 ± 9.4*	85.4
	Saline	7	Vo	—	156.1 ± 15.6	—
Ethanol ^b	CHE	5	Sc	700	95.1 ± 40.8*	40.2
	Carbenoxolone	7	Sc	200	113.7 ± 21.1*	28.5
	Saline	7	Sc	—	158.9 ± 42.7	—
Stress ^c	CHE	5	Vo	1000	9.0 ± 1.7*	82.3
	Cimetidine	7	Vo	100	15.0 ± 1.4*	70.5
	Saline	7	Vo	—	50.8 ± 21.7	—
Reserpine ^d	CHE	5	Vo	1000	11.8 ± 5.4*	53.9
	Atropine	5	Vo	10	12.6 ± 3.9*	50.9
	Saline	6	Vo	—	25.7 ± 8.5	—

^aANOVA $F_{(2,18)} = 267.5$ $p < 0.001$. Duncan's test * $p < 0.001$.

^bANOVA $F_{(2,16)} = 5.53$ $p < 0.05$. Duncan's test * $p < 0.05$.

^cANOVA $F_{(2,12)} = 19.64$ $p < 0.001$. Duncan's test * $p < 0.001$.

^dANOVA $F_{(2,14)} = 8.82$ $p < 0.01$. Duncan's test * $p < 0.01$.

Table 2. Effect of intraduodenal administration of the CHE, coumarin **1** and cimetidine, in the pyloric ligation model in rats

Model	Treatment	Dose (mg/kg body wt.)	<i>n</i>	Volume (ml)	[H ⁺] (mEq/l)
Pyloric ligation ^a	Saline	—	5	5.5 ± 1.3	1.7 ± 0.4
	Cimetidine	100	5	4.2 ± 1.5	1.3 ± 0.2*
	CHE	1000	5	1.3 ± 0.2**	0.8 ± 0.6*
	Coumarin 1	100	5	2.7 ± 1.6*	0.9 ± 0.1*
Pyloric ligation (histamine) ^b	Saline	—	5	2.8 ± 1.4	1.9 ± 0.3
	Cimetidine	100	5	1.0 ± 0.4*	1.2 ± 0.5*
	CHE	1000	5	2.1 ± 0.4	1.9 ± 0.3
	Coumarin 1	100	5	1.9 ± 0.8	1.8 ± 0.1
Pyloric ligation (pentagastrin) ^c	Saline	—	5	3.3 ± 1.0	1.1 ± 0.5
	Cimetidine	100	5	1.9 ± 0.6*	0.5 ± 0.2*
	CHE	1000	5	1.8 ± 0.9*	1.3 ± 0.5
	Coumarin 1	100	5	2.0 ± 0.7*	1.08 ± 0.6
Pyloric ligation (bethanechol) ^d	Saline	—	5	10.8 ± 3.5	1.3 ± 0.5
	Atropine	10	5	0.6 ± 0.2**	0.7 ± 0.2*
	CHE	1000	5	3.8 ± 0.5**	0.8 ± 0.3*
	Coumarin 1	100	5	3.7 ± 1.0**	0.8 ± 0.1*

^aANOVA: Volume: $F_{(3,16)} = 10.26$ $p < 0.001$; H⁺: $F_{(3,16)} = 5.85$ $p < 0.01$. Duncan's test * $p < 0.01$ ** $p < 0.001$.

^bANOVA: Volume: $F_{(3,16)} = 3.53$ $p < 0.05$; H⁺: $F_{(3,16)} = 3.04$ $p < 0.05$. Duncan's test * $p < 0.05$.

^cANOVA: Volume: $F_{(3,16)} = 5.16$ $p < 0.01$; H⁺: $F_{(3,16)} = 5.85$ $p < 0.05$. Duncan's test * $p < 0.01$.

^dANOVA: Volume: $F_{(3,16)} = 18.04$ $p < 0.001$; H⁺: $F_{(3,16)} = 4.17$ $p < 0.05$. Duncan's test * $p < 0.05$, ** $p < 0.001$.

Histamine is produced from mast cells or histamine-containing cells similar to mast cells, which lie close to the parietal cells. There is a steady basal release of histamine, which can be increased by gastrin and acetylcholine. The parietal cells have H₂-receptors for histamine and, when stimulated, increase cAMP that will activate the protein kinase A. This enzyme will trigger sequential events that lead to acid secretion (Garcia et al., 1978; Solcia et al., 1993).

After histamine administration (25 mg/kg body wt., sc) neither CHE (1000 mg/kg body wt.) nor coumarin (100 mg/kg body wt.) altered the gastric juice volume or hydrogenionic concentration (Table 2). These results suggested that they did not block the H₂ receptors at gastric parietal cells or influence the intracellular mechanisms involved with histamine activity.

Pentagastrin is the functional extremity of the gastrin molecule, with a β-alanine substitute (Ding and Hakanson, 1996). This substance acts on the CCK-2 gastrin receptor (Schubert, 2000), increasing acid liberation and also stimulating histamine secretion of enterochromaffin cells (Hersey and Sach, 1995; Andersson et al., 1996; Angus and Black, 1982). When this secretagogue was administered intravenously in advance (4 μg/kg body wt.), both CHE (1000 mg/kg body wt.) and coumarin **1** (100 mg/kg body wt.) produced a reduction of 46% and 61%, respectively, of the volume, but did not alter the gastric acid hydrogenionic concentration secretion (Table 2).

The vagus nerve stimulates stomach acid secretion via interaction of their chemical mediator (acetylcholine) with muscarinic receptors. According to some authors,

those receptors are located at parietal cells and at histamine secretory cells. So the acid secretion increase is a consequence of acetylcholine activity on histamine cell and on parietal cell activity (Barocelli et al., 1997; Schubert, 2000). Bethanechol is a selective agonist for muscarinics that increases gastric acid secretion (Schubert, 1994; Schubert, 2000).

In animals treated subcutaneously with bethanechol (1.5 mg/kg body wt.), CHE and coumarin **1** reduced the volume by 64% and 77%, respectively, with 41% and 58% reductions of hydrogenionic concentration (Table 2). The activation of the muscarinic receptor present in the parietal cell gives a start to sequential events, triggered by G-protein, that lead to inositol triphosphate and diacylglycerol liberation from the membrane phospholipid fraction. Protein kinase C, activated by diacylglycerol, increases gastric acid secretion and inositol triphosphate increases intracellular calcium release as well as chloridric acid secretion (Clapham, 1995).

The blockage of bethanechol activity by CHE and coumarin **1** suggested an anticholinergic mechanism or an interruption of intracellular events that are linked to acid secretion. The parasympathetic agents induce bronchial muscle relaxation and also decrease bronchial secretion. Since something similar may be happening with CHE and coumarin, it is probable that the same pharmacological mechanism is involved in both anti-ulcerogenic and bronchodilator activity. This might also explain the bronchodilation effect described for folk medicine, because cholinergic mechanisms are involved in bronchial contraction and secretion.

Coumarin **1** increased the specific activity tenfold, suggesting that it is the main compound responsible for the CHE gastric secretion decrease. Another species from this genus *M. cordata* showed antiulcerogenic activity (Rabin et al., 2000). According to the authors, however, the active principles are alkaloids that are not found in *M. laevigata*.

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