

Healing Process Induced by a Flavonic Fraction of *Bidens aurea* on Chronic Gastric Lesion in Rat. Role of Angiogenesis and Neutrophil Inhibition

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The aim of this study was to elucidate the mechanism of the healing process mediated by the flavonoid fraction of *Bidens aurea* on chronic gastric ulceration induced by 5% acetic acid in rats. The diethyl ether extract (125 and 62.5 mg kg⁻¹ body weight) was administered in a single doses, 7 and 14 days after provocation of lesions. Our results demonstrated that both doses significantly decreased the macro and microscopic ulcer index. Usually after 14-days treatment the lesions were found completely covered with regenerative epithelium and also an important proliferation of blood vessels was observed. Myeloperoxidase (MPO) activity was assayed and used as an index of leucocyte infiltration. Application of acetic acid produced a significant increase of this activity 7 days after induction of chronic injury. Administration of 125 mg kg⁻¹ of the ether extract provoked a sharp reduction on the enzymatic activity at the same period. After 14 days, this decrease was higher with both doses ($p<0.001$). In addition, the macroscopic examination showed a drastic reduction of leucocyte infiltration in treated groups. These results suggest that the recovery of vascularization of the ulcerated area and the decrease of neutrophil infiltration are involved in the antiulcerogenic effect of the flavonoid fraction of *Bidens aurea*.

Introduction

It is well known that flavonoids have a range of biological properties and pharmacological effects, including pronounced antiulcerogenic activity (Barnaulov *et al.*, 1983; Konturek *et al.*, 1986; Rainova and Nakov, 1988; Martín *et al.*, 1988). *Bidens aurea* (Aiton) Sherff is a species with a high content of flavonoid compounds, mainly aurones and chalcones (La Casa *et al.*, 1995). In previous reports our work group has demonstrated the antiulcerogenic effect of the diethyl ether extract of this plant against different experimental models, namely stress by immobilisation and cold (Alarcón de la Lastra *et al.*, 1994) and necrotizing agents (La Casa *et al.*, 1995).

The few studies on the healing effect of these polyphenolic substances in long-range treatments (Parmar and Ghosh, 1981; Barnaulov *et al.*, 1982) has prompted us to investigate the effect of the flavonoid extract of *B. aurea* on experimental

chronic ulcers in rats. The Okabe and Pfeiffer model (1971), using 5% acetic acid, induces gastric lesions to resemble the human peptic ulcer, both macroscopically and histologically.

The study has been performed to determine the effects of the ether extract residue on chronic mucosal lesions. We have studied the evolution of the healing process of the ulcer by macroscopic and histological measurements. Recently, neutrophils have been implicated in the pathogenesis of several types of injury to the gastrointestinal tract, including the ulceration induced by haemorrhagic shock (Smith *et al.*, 1987), ethanol (Kvietys *et al.*, 1990), ischemia-reperfusion (Hernandez *et al.*, 1987), non-steroidal antiinflammatory drugs (Wallace *et al.*, 1990; Wallace and Granger, 1992), and acetic acid (Motilva *et al.*, 1995). Thus, we have also investigated the changes that the flavonoid fraction exerts on mucosal neutrophil infiltration.

Materials and Methods

Plant material and extraction procedures

Flowery tops of *Bidens aurea* (Aiton) Sherff, Asteraceae, were collected at Puerto Serrano (Cá-

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diz, Spain) in November 1992 and were identified at the Botany Laboratory of the Faculty of Pharmacy of Sevilla University (Spain). Voucher specimens are kept under reference SE-VS. The plant samples were air dried at room temperature, 15–21°C.

The material (500 g) was extracted successively by Soxhlet, first with chloroform (2 l, 99% Panreac, Barcelona, Spain) and then with methanol (2 l, 99% Panreac, Barcelona, Spain). The solvent was removed under vacuum in a rotary evaporator. The dry methanol residue (32.96 g) was partitioned by the Netien Lebreton method (1964), to produce the diethyl ether and ethyl acetate extracts which were dried under vacuum. Both extracts were obtained with diethyl ether (99%) and ethyl acetate (98%) (Panreac, Barcelona, Spain) respectively. Since in a previous report (Alarcón de la Lastra *et al.*, 1994) the ether extract proved the most effective, it was chosen for gastric studies.

Animals groups and drug preparation

Male and female Wistar rats (supplied by Animal Service, Faculty of Pharmacy, University of Seville) weighing 180–200 g were used throughout this study. They were fed a normal laboratory diet and given tap water to drink. The temperature was maintained at 22–24°C and humidity at 70–75% in a controlled room.

Dry ether extract residue (125 and 62.5 mg kg⁻¹ body weight), was given orally in aqueous suspension with Tween 20 (1%). Control rats received the aqueous vehicle in a comparable volume (1 ml/100 g, v.o.).

Induction of chronic gastric injuries

Gastric lesions were induced according to the method described by Okabe and Pfeiffer (1971). Briefly, rats were superficially anaesthetized with diethyl ether and their stomachs exposed through medial laparotomy. The antral areas of the stomachs were injected with 0.05 ml of 5% acetic acid. This procedure made possible the production of histologically characteristic ulcers, 2–3 days after this injection, with a benign spontaneous evolution (Okabe and Pfeiffer, 1971; Navarro *et al.*, 1990). Treatment was started 24 h before the procedure and extended for 7 and 14 days after surgical intervention.

Macroscopic and histological evaluation of gastric damage

Animals, 6–8 for each group, were sacrificed at 7 and 14 days after acetic acid injection. The stomach was exposed and an incision was made in the duodenum. A polyethylene canula was placed into the stomach via oesophagus, and the gastric lumen was washed with 10–15 ml saline. To maintain a similar intragastric pressure in all animals, the same amount of formol buffered fixative solution (10%, pH 7.0) was perfused through a cannula inside the gastric cavity. After the solution had emptied through the duodenal incision, the esophagus and duodenum were ligated. Fifteen minutes later, the stomach was removed and placed in the same fixative solution for 24 h. At the end of this period, the stomach was cut along its greater curvature and the length and width of gastric ulcer were measured determining the lesion index (UI, mm²); the central part of the ulcerated tissue was cut in half along the long diameter. The tissue was then embedded in paraffin. Four serial sections of 5–7 µm thick were taken, stained with haematoxylin and eosin (HE), and examined microscopically (Carl Zeiss II Microscope). For each section the following parameters were noted.

Gastric damage

The stained sections were quantified according to the scale proposed by Bulbena *et al.* (1991) with some modifications: 0 no lesions; 1 minimal lesion or slight oedema of the luminal surface of the mucosa; 2 lesion completely covered by well defined mucosecretor epithelium, little inflammatory response, significant penetration of granulation tissue with typical arrangement of fibroblast and collagen fibres and an important degree of vascularization; 3 lesion covered by mucosecretory epithelium, less development of granulation tissue, medium degree of vascularization; 4 epithelialization at the margins of the wounded area, necrotic layer covering the lesion, slight granulation tissue development and vascularization; 5 severe erosion of the mucosa without any newly formed mucosecretory epithelium, widespread necrotic tissue covering the lesion, strong inflammatory response; no revascularization.

Presence of regenerative epithelium

Development of regenerated epithelium was evaluated using the following scale (Hernandez *et al.*, 1987): 0 absence; 1 slight presence; 2 medium presence; 3 severe proliferation; 4 completely regenerated.

Infiltration of inflammatory cells

The severity of infiltration was graded from 0 (no infiltration) to 4 (marked inflammatory infiltration).

Grade of angiogenesis

The development of vascularization was assessed using the following score: 0 no vascularization; 1 slight presence; 2 mild presence; 3 elevated presence of blood vessels.

For each animal, the median value derived from the four slides taken was used for analysis. The assessment was made by a pathologist unacquainted with the experimental findings. When required, photographs were taken using an Olympus OM-UTI camera.

Assessment of leukocyte involvement

Neutrophil infiltration in vivo was assessed by measuring granulocyte specific enzymes such as myeloperoxidase (MPO) in tissue (Grisham *et al.*, 1990; Komatsu *et al.*, 1992). Myeloperoxidase activity in this experimental model was measured on days 7 and 14 after ulcer induction in new groups of animals. A new control group was also treated with vehicle, but acetic acid was not injected (control, C).

Tissue preparation

Two samples, one from gastric lesion and the other from the undamaged symmetrical zone to the lesion, were obtained. Samples were excised from each animal and rapidly rinsed with ice-cold saline, blotted dry, and frozen at -70°C. The assay for myeloperoxidase activity was always performed within 2 weeks of an experiment. The tissue was thawed, weighed, and homogenized in 10 volumes of 50 mM potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 20000×g, 20 min, 4°C. The pellet was again homogenized in 10 volumes of 50 mM potassium

phosphate buffer (pH 6.0) containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM EDTA. The homogenate was subjected to one cycle of freezing/thawing and a brief period of sonication (30 sec).

Myeloperoxidase assay

Briefly, myeloperoxidase activity was assayed spectrophotometrically using a minor modification of the method, which utilizes 3,3',5,5'-tetramethylbenzidine (TMB) as substrate (Grisham *et al.*, 1990; Komatsu *et al.*, 1992). In this method 0.5 µl of homogenate is added to a 0.5 ml reaction volume containing 80 mM phosphate buffered saline (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 H₂O₂. Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37°C. The reaction was terminated by the sequential addition of catalase (20 µg/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 myeloperoxidase activity was defined as the amount of enzyme present that produced a change in absorbance per minute of 1.0 at 37°C in the final reaction volume containing the acetate.

Statistical analysis of data

The data are presented as the mean±ESM, and the statistical analysis employed has been the Mann-Whitney U-test (changes in ulcer index, UI) and the Student's t-test for unpaired data (for the rest of experiences).

Results

1 - Effects of ether extract on gastric damage induced by acetic acid

The lesions were macroscopically examined in all animals at 7 and 14 days after acetic acid application. The ulcer index values seen in controls were 30.5±3.5 mm² on day 7 and 10.7±1.7 mm² on day 14 (p<0.001), indicating that the ulcers spontaneously diminished in size (Fig. 1). The ether extract treatment (125 mg kg⁻¹) for 7 days induced a significant decrease of ulcer index, 4.34±0.8 mm² (p<0.001) and this effect was higher

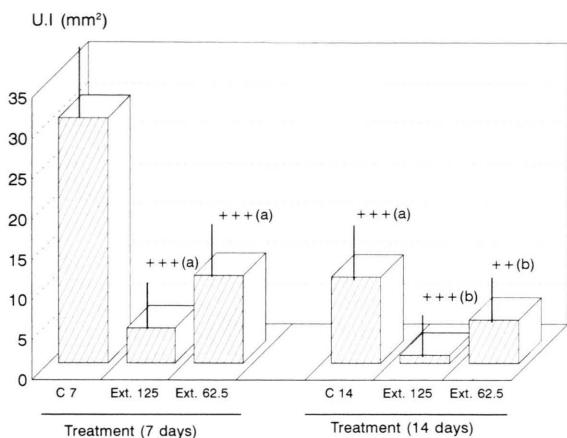


Fig. 1. Ulcer index (UI) of control groups 7 (C7) and 14 (C14) days after acetic acid application. Effect of ether extract of *Bidens aurea* (Ext.125 and Ext.62.5 mg kg⁻¹ s.o.) at same periods.

Mann-Whitney U-test: +++ p<0.001 v.s C 7 (a) and v.s C 14 (b).

after 14 days with both doses, 62.5 and 125 mg kg⁻¹ (5.4±0.7, p<0.01 and 1.0±0.1, p<0.001 respectively).

These results were confirmed by microscopic examination. After 7 days of treatment, a disorganization of the histological layers which make up the stomach wall was observed, and also an area with necrotic tissue and a large quantity of neutrophils appeared. The glands on the margins were dilated and there was an increase in the mucus secretion of the isthmus and neck, but one may speak of epithelium regeneration with mucous cells containing a large amount of granules. The severity of chronic ulceration as judged by microscopy ranged from 3.82±0.59 at day 7 to 2.21±0.28 at day 14 (p<0.01) in the control groups (Table I). The mor-

phology of the microscopic lesion (range of regenerative epithelium) varied from severe erosion of the mucosa without newly-formed mucosecretory tissue (1.56±0.28) at day 7, to lesions covered by mucosecretory cells but without a well structured gastric wall (3.01±0.12) at day 14, p<0.05. In the animals treated with ether extract for 14 days it was usual to find the lesions completely covered by regenerative tissue, sometime arranged into columnar structures above granular tissue, with significant degree of vascularization (regenerative epithelium degree: 125 mg kg⁻¹, 2.87±0.36 and 62.5 mg kg⁻¹, 2.56±0.27, p<0.05). An important proliferation of blood vessels was observed in the internal area of the ulcerative formation (2.4 ± 0.4, p<0.01).

2 – Effect of myeloperoxidase activity, MPO, and grade of infiltrating polymorphonuclear leucocytes (PMN)

Using myeloperoxidase activity as an index of neutrophil infiltration, we found that serosal application of acetic acid produced an increase in the lesioned area of the day-7 group which was consequently significantly larger than that of the control group, p<0.001 (Fig. 2). The administration of ether extract, 125 mg kg⁻¹, provoked a sharp reduction of MPO activity in the same period 7 days (14.16±0.75 U mg⁻¹ ulcerated tissue, p<0.001). After 14 days, this decrease was higher with both doses, p<0.001 (Fig. 3). However, the enzymatic activity was not modified on the undamaged mucosa in the same period.

These results agreed with those found under microscopic examination (Table I), which showed a

Table I. Histological evaluation of acetic acid-induced ulcers after 7 and 14 days of treatment with ether extract of *B.aurea*.

Treatment [mg/kg]	Ulcer index	Regenerative epithelium	Inflammatory infiltration PMN	Angiogenesis grade
Control without acetic acid				
Control 7 days	3.82 ± 0.59	1.56 ± 0.28	2.41 ± 0.31 *** (a)	1.0 ± 0.3
Ether extract 125 mg/kg (7 days)	2.67 ± 0.32 *(b)	1.96 ± 0.19	1.24 ± 0.32 *** (a) *(b)	1.6 ± 0.3
Ether extract 62.5 mg/kg (7 days)	3.43 ± 0.19	2.07 ± 0.30	2.36 ± 0.29 *** (a)	1.2 ± 0.4
Control 14 days	3.01 ± 0.28	2.21 ± 0.42 *(b)	2.10 ± 0.12 *** (a)	2.0 ± 0.5 *(b)
Ether extract 125 mg/kg (14 days)	1.93 ± 0.09 ** (b)	2.87 ± 0.36 *(b)	1.12 ± 0.09 *** (a)	2.4 ± 0.4 ** (b)
Ether extract 62.5 mg/kg (14 days)	2.64 ± 0.12 *(b)	2.56 ± 0.27 *(b)	1.56 ± 0.23 *** (a)	2.1 ± 0.1 *(b)

Student-Fisher t-test: * p < 0.05, ** p < 0.01, *** p < 0.001 vs control (a) vs control 7 (b).

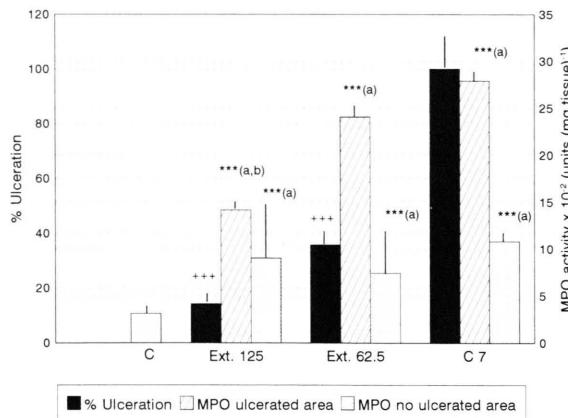


Fig. 2. Myeloperoxidase activity (MPO) obtained from damaged and non-damaged areas in chronic gastric ulcerated groups (percentage of ulceration) with ether extract of *Bidens aurea* treatment (Ext.125 and Ext.62.5 mg kg⁻¹) after 7 days (C 7). C, no ulcerated control group.

Mann-Whitney U-test: + + + p < 0.001 v.s C 7.
Student's t-test: *** p < 0.001 (a) v.s. C and (b) v.s. respective control group after 7 days of treatment C 7.

greater infiltration of inflammatory cells in the day 7-group (2.41 ± 0.31 , p < 0.001) and a drastic reduction in treated groups after 7 (125 mg kg⁻¹) and 14 days (125 and 62.5 mg kg⁻¹), p < 0.001.

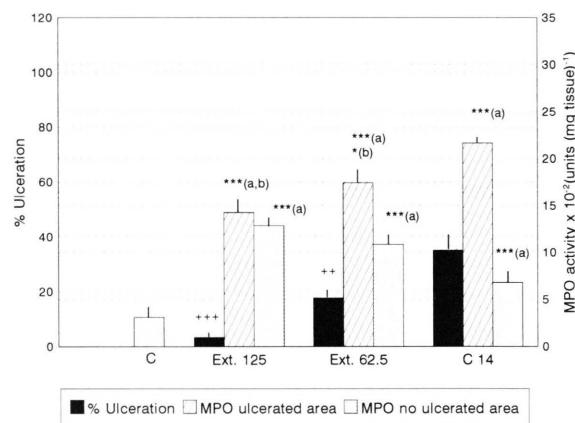


Fig. 3. Myeloperoxidase activity (MPO) obtained from damaged and non-damaged areas in chronic gastric ulcerated groups (percentage of ulceration) with ether extract of *Bidens aurea* treatment (Ext.125 and Ext.62.5 mg kg⁻¹) after 14 days (C 14). C, no ulcerated control group. Mann-Whitney U-test: + + + p < 0.001, + + p < 0.01 v.s C 14.

Student's t-test: *** p < 0.001 * p < 0.05 (a) v.s. C and (b) v.s. respective control group after 14 days of treatment C 14.

Discussion

Chronic gastric lesions by acetic acid in rats are known to resemble the human peptic ulcer, both grossly and histologically. The model of Okabe and Pfeiffer (1971) is widely used for studying the effect of drugs on healing rates. In this experimental model, after a period of 17 days, the macroscopic gastric lesions were allowed to heal spontaneously in 50% of all animals examined (Navarro *et al.*, 1990; Ogihara and Okabe, 1993).

In our experiments we have observed the same effect, since the percentage of ulceration decreased from 100% (7 days) to 35.1% (14 days). However, this healing process was significantly higher in the stomach of the animals treated with ether extract of *Bidens aurea*. In these groups the ulcer was covered with tissue of a similar colour and appearance to that of the adjoining glandular areas, and was related to a highly advanced cure process. This was confirmed microscopically, since an elevated epithelialization was seen in the majority of the stomachs at the end of treatment. The internal area of the ulcerative formation was composed of a lax connective tissue with a typical arrangement of fibroblasts and collagen fibres. Some eosinophilic leucocytes and an important vascularization were also observed.

These results seem to indicate that the treatment with ether extract induces angiogenesis in the erosionated areas. It is well known that the regulation of microcirculation may be an essential event in the protection mechanisms of the gastric mucosa (Whittle, 1989). Correct gastroduodenal blood flow is very important for the normal working of the gastric processes. It was demonstrated that the recovery of the vascularization of the ulcerated area was necessary in the mucosal regeneration processes (Kamada *et al.*, 1983). Angiogenesis within granulation tissue is considered one of the most necessary processes in ulcer healing (Tarnawski *et al.*, 1991). Recently, a parallelism has been established between angiogenesis and the curative process. Motilva *et al.* (1992), in a similar experimental model, using acetic acid 20%, have demonstrated that the proliferation of blood vessels induced by naringenin, in the internal area of ulcerative formation, could explain the regeneration of the gastric mucosa in the animals treated with this flavonoid. In contrast, the reduction of

blood flow resulted in ischemia, which could generate cellular necrosis.

Probably, the healing process induced by *B. aurea* could be partly associated with its capacity to stimulate vessel proliferation.

In the last years, the role of neutrophils has been examined in gastropathy. The activation of these leucocytes by different methods, ischemia-reperfusion (Hernandez *et al.*, 1987), non-steroidal anti-inflammatory drugs (Wallace *et al.*, 1990), haemorrhagic shock (Smith *et al.*, 1987), causes adherence to the vascular endothelium, probably through the release of inflammatory mediators that increase the expression of adhesion molecules on the cellular surface (Wallace *et al.*, 1990; Asako *et al.*, 1992a,b). Adherence of these leucocytes to the endothelium result in reduced perfusion of the mucosa, which predisposes it to injury. Activation of these cells as a consequence of stimulation by inflammatory mediators, leukotriene B₄, platelet-activating factor (Wallace, 1989; Vaananen *et al.*, 1992), and their adherence, results in the release of tissue-damage factors such as oxygen-derived free radicals. These factors damage the endothelium and possibly other cells in the mucosa (Vaananen *et al.*, 1991).

Recent studies indicate that these leucocytes are also involved in the pathogenesis of acetic acid-induced chronic ulcers. Motilva *et al.* (1995) and Martín *et al.* (1995), have demonstrated that this agent induces a significant increase of PMN infiltration in the gastric mucosa which is related to a parallel increase of MPO activity. In addition, in the animals that received hydroxyurea, a leucopenic agent, the ulcer index and MPO activity were significantly reduced. In the same

way, depletion of the number of PMNs by treatment with methotrexate and dexametasone significantly diminished the exacerbation of gastric injury after ischaemia and reperfusion (Alarcón *et al.*, 1995). Also, prevention of neutrophilic adherence to the vascular endothelium through treatment with an antibody directed against the β_2 integrin CD18 resulted in nearly complete protection of the gastric mucosa (Wallace *et al.*, 1991). Our results suggest that acetic acid induced impairment of mucosal functions may be partly mediated by the products of neutrophil activation, e.g. reactive oxygen metabolites (ROM) and cytotoxic proteins.

In a previous study (La Casa *et al.*, 1995), phytochemical analysis of the ether extract showed the presence of polyphenolic compounds, mainly aurones and chalcones, and genins maritimetin and sulfuretin could be identified by spectroscopic and chromatographic methods. Several compounds of this nature have shown antiulcer activity in different experimental models (Kygoyu *et al.*, 1979; Hatayama *et al.*, 1985). In addition, numerous flavonoids exert significant scavenging properties on ROM "in vivo" (Robak *et al.*, 1988; Bors *et al.*, 1994) and "in vitro" (Morel *et al.*, 1994) and these antioxidant effects have been related to their antiulcer activities (Martín *et al.*, 1994, Pérez-Guerrero *et al.*, 1994).

Thus, it is possible that the ether extract of *B. aurea* exerts its healing effect in this experimental model through a complex mechanism including the recovery of the vascularization of the ulcerated area and a decreasing of neutrophil infiltration, and therefore of the neutrophil-mediated cytotoxicity.

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