

Role of Mucus Secretion and Sulfhydryl Groups in Gastroprotection Mediated by a Flavonoid Fraction of *Bidens aurea*

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The aerial parts of *Bidens aurea* (Aiton) Sherff have been used in folk medicine as an antiulcer agent. The efficacy of an ether extract to prevent gastric mucosal damage produced by several necrotic agents was studied in rats. The best efficiency was against lesions induced by 30% NaCl with a significant decrease of damage at all doses tested (250, 125 and 62.5 mg kg⁻¹). In contrast, only the highest dose (250 mg kg⁻¹) was effective on gastric erosions induced by 0.6 N HCl. This effect could not be related with the mucus gel production only since the groups treated with of absolute ethanol or 30% NaCl before and then received 250 mg kg⁻¹ of the ether extract increased significantly the amount of gastric mucus and its glycoprotein content. However, the ether extract of *Bidens aurea* enhanced the production of mucosal non-protein SH groups at all doses assayed after treatment with absolute ethanol and 0.6 N HCl. With NaCl-induced gastric injury, also the highest dose (250 mg kg⁻¹) produced an increase of this parameter. The results suggest that a slight augmentation of the mucus gel concentration and the increase of non-protein sulfhydryl compounds, could contribute to the functional protection mechanism mediated by a flavonoid fraction of *B. aurea* in these experimental models.

Introduction

Several flavonoids prevent gastric mucosal lesions produced by various methods of experimental ulcer and protect the gastric mucosa against various necrotic agents (Martin *et al.*, 1994; Motilva *et al.*, 1994; Pérez Guerrero *et al.*, 1994). Some of them have been shown to increase the mucosal content of prostaglandins (PGs) and mucus gel in gastric mucosa, showing cytoprotection effects (Motilva *et al.*, 1994).

Although the mechanism of gastric mucosal protection against injury is multicomponential in nature, the initial brunt of luminal insult falls on the layer of mucus, which constitutes the only identifiable physical barrier between the gastric lumen and the surface epithelial cells of the mucosa (Allen *et al.*, 1993). Enhancement of the gastric mucus has been proposed to explain the antiulcer

activity of different compounds. If the layer is thick enough and is renewed frequently, it may have an important function as a barrier (along with bicarbonate) against aggressive agents (Allen *et al.*, 1993; Wallace and Whittle, 1986).

Bidens aurea (Aiton) Sherff, Asteraceae, is a species with a high content of polyphenolic compounds, mainly flavonoids and tannins. In previous reports our work group have demonstrated the anti-ulcerogenic effect of different extracts of this plant, an ether extract being the most effective (Ayuso *et al.*, 1986; Alarcón de la Lastra *et al.*, 1994).

These reports prompted us to study in some depth the ability of this active extract to prevent gastric injury produced by necrotizing agents which induce hemorrhagic lesions. In addition, the present investigation was designed to examine the gastric mucus content and composition, since gastric mucus is considered to be a physiological barrier which plays an important role in protecting the gastric mucosa.

It is also known that the cytoprotective effect of several drugs is partly mediated by an enhancement of non protein-sulfhydryl groups (NP-SH) in

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the gastric mucosa (Szabo, 1989). Reduced levels of endogenous sulfhydryls have been associated with tissue damage (Miller *et al.*, 1985; Tariq *et al.*, 1987). Therefore, we investigated the possible role of SH-compounds in the gastroprotection mediated by an ether extract. We also proceeded to identify the constituents that could be involved in its pharmacologic action.

Material and Methods

Plant material

Flowerly tops of *Bidens aurea* (Aiton) Sherff, Asteraceae, were collected at Puerto de Santa María (Cádiz, 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.

Extraction and isolation procedures

The material (500 g) was extracted successively, by Soxhlet, first with chloroform (99%, Panreac, Barcelona, Spain) and then with methanol (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 method of Netien and Lebreton (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. The ether extract was chosen for gastric cytoprotective studies. This extract was fractionated by column chromatography (CC) on silica gel with n-hexane/AcOEt/MeOH mixtures. The fractions containing the predominant flavonoids were further purified by Sephadex LH20 column with $\text{Cl}_2\text{CH}_2/\text{MeOH}$ 1:1 (v:v). Chalconic and auronic compounds were separated by HPLC (Hewlett Packard 1050), an instrument equipped with a multisolvent delivery system (Model 600E) and a programmable photodiode array detector (Model 994). A prepacked analytical column (25 cm x 4 mm, 5 μm) of Spherisorb ODS-2 RP-18 (Supelco) was used for analysis. Two solvents were used, 1% aqueous acetic acid (99%, Panreac, Barcelona, Spain) and methanol (HPLC grade, 99.8%, Pan-

reac, Barcelona, Spain). The analysis was carried at room temperature and the flow rate was 1 ml/min at an average column pressure of 2,000 psi. The chromatograms were monitored at 254 nm.

Animals groups and drug preparation

Wistar rats of both sexes, weighing 180–250 g were fed with standard diet. Prior to the experiments, the rats were deprived of food for 24 h and kept in raised mesh-bottomed cages to prevent coprophagy. Drinking water was allowed ad libitum.

The animals were randomly assigned to groups: experimental groups were usually treated with three doses, 250, 125 and 62.5 mg kg⁻¹ body weight of a diethyl ether extract. Carbenoxolone (Leo S.A., Madrid, Spain) was used as standard drug, 80 mg kg⁻¹. The agents were dissolved in Tween 20 (1%) and were administered by intragastric route in a ratio of 1 ml/100 g body weight. Control groups received vehicle in comparable volume.

Protection against necrotizing agents

Ulceration was induced by instillation of 1 ml of the necrotizing agents 0.6 N HCl, absolute ethanol or 30% NaCl (Robert, 1979). The drug was administered in different doses to different groups of animals one hour before the *p.o.* administration of the necrotizing agents. Two hours after the experimental period, the animals were sacrificed using an overdose of diethyl ether and their stomachs were removed and opened along the greater curvature. Any lesions present were examined macroscopically. The number of erosions per stomach was assessed for severity according to our score system: 0) no lesions; 1) one or more hemorrhagic ulcers length <5 mm; 2) one hemorrhagic ulcer length >5 mm and thin; 3) more than one ulcer grade 2; 4) one ulcer length >5 mm and width >2 mm; 5) two or three ulcers of grade 4; 6) from four to five ulcers of grade 4; 7) more than six ulcers of grade 4; 8) complete lesion of the mucosa. Mean scores for each group were calculated and expressed as ulcer index (UI). The percentages of mucosal ulceration were also expressed.

Biochemical study of gastric mucus

The gastric mucus was obtained by scraping off the mucosa with a glass slide and was immediately

homogenized in 4 ml of distilled water. The weight of mucus (g) was the difference between the weight of homogenate and that of the original 4 ml of water (Bulbena *et al.*, 1986). In order to avoid variations in either the criteria of gastric mucosal lesion or scraping technique, they were preformed by the same person.

Total proteins (mg ml⁻¹) were determined from one portion of the homogenate (1ml), following the colorimetric technique of Lowry *et al.* (1951). The hexosamine content (µg ml⁻¹) was determined from method described by Boas (1953).

Determination of mucosal sulfhydryls

The amount of mucosal sulfhydryls was measured in the gastric mucosa according to the method described by Garg *et al.* (1991). The doses of ether fraction were administered to new groups of animals 120 min before the administration of absolute ethanol. One hour after the experimental period, the rats were sacrificed using an overdose of anaesthetic and their stomachs removed, opened, rinsed in ice-cold sodium phosphate buffer, homogenized, made up to 2 ml with buffer and centrifuged at 3800×g for 10 min at 4°C. The supernatant sulfhydryl content was determined. Light absorbance at 412 nm, against a reagent blank, was measured with a spectrophotometer (Perkin-Elmer Lambda 3). Sulfhydryl concentrations, calculated from freshly prepared standard curves of glutathione (Sigma Chem. Co., St.Louis, Mo.) were expressed as µmol/g tissue.

Statistical analysis

Values are given as arithmetic means ± ESM. Differences between two groups were analyzed using the Mann-Whitney U-test (changes in ulcer index, UI) and the Student's *t*-test for unpaired data (the rest of the experiences).

Results

Phytochemical analysis

Phytochemical analysis of the ether extract of *Bidenes aurea* shows the presence of polyphenolic compounds mainly aurones and chalcones, and the genins maritimetin and sulfuretin were identified by spectroscopic methods (UV) in comparison with the data of authentic samples and published

results (Mabry *et al.*, 1970; Pardo and Fernandez, 1978). HPLC quantitative analysis was performed by the relative proportions method (areas) under the conditions specified in the material and methods. In these experimental conditions, maritimetin was a predominant compound.

Effects of ether extract and carbenoxolone on ulcer induced by necrotizing agents

Treatment of control groups with ethanol produced an ulcer index of 7.0 ± 0.3 ($n=6$). Pretreatment of the animals with the ether extract at doses of 250 and 125 mg kg⁻¹, significantly reduced the ulcer index to 3.0 ± 0.01 ($p<0.01$) and 3.6 ± 0.40 ($p<0.05$), respectively (Fig.1).

The protective effect against gastric lesions induced by 30% NaCl, was statistically significant ($p<0.001$ and $p<0.05$) at all doses tested (Fig.2).

By contrast, only the highest dose, 250 mg kg⁻¹, reduced ($p<0.01$) the ulceration induced by 0.6 N HCl (Fig.3).

In addition, the percentages of mucosal ulceration obtained after treatment with 250 mg kg⁻¹ of ether extract were 42.8% (absolute ethanol), 36.3% (30% NaCl) and 55.1% (0.6 N HCl). In absolute ethanol and 30% NaCl ulcers, these percentages were lower than that of carbenoxolone (80 mg kg⁻¹) (Fig.4).

Effect of ether extract and carbenoxolone on gastric mucus gel content in rats treated with necrotizing agents

The group that received 250 mg kg⁻¹ of ether extract and then 1 ml absolute EtOH or 30% NaCl, increased significantly the amount of mucus gastric gel (1.24 ± 0.01 , $p<0.001$ and 0.77 ± 0.20 $p<0.05$ respectively) and their glycoprotein content (Tables I and II), compared to control groups. This increase was higher than that of carbenoxolone (80 mg kg⁻¹).

In contrast, the treated groups with 0.6 N HCl showed no significant changes in the amount of mucus, total proteins and hexosamines content (Table III).

Effect of ether extract and carbenoxolone on SH-groups content in rats treated with necrotizing agents

In normal rats the levels of non-protein and total SH-groups in the gastric mucosa were $0.531 \pm$

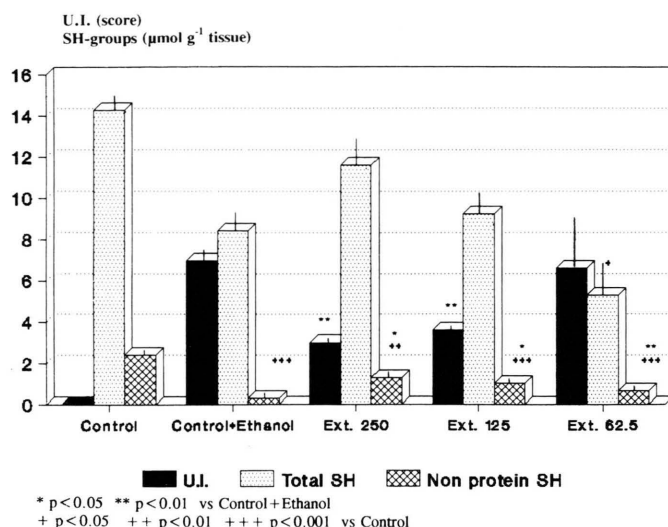


Fig. 1. Effects of the ether extract of *Bidenes aurea* on lesions induced by absolute ethanol (ulcer index, UI) and changes in mucosal sulfhydryl groups content (120 min).

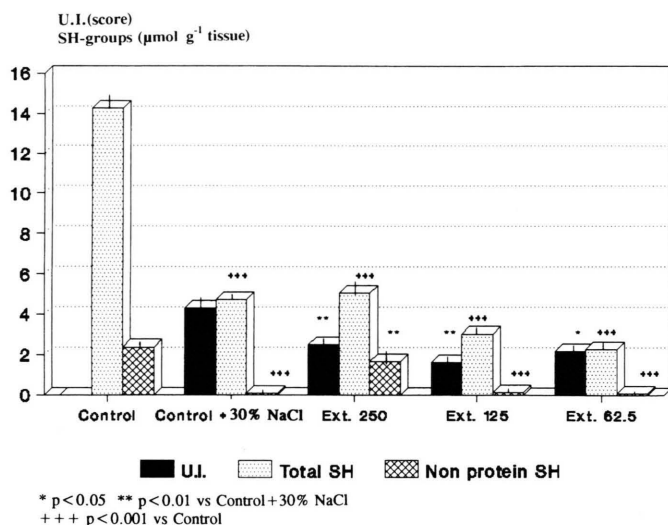


Fig. 2. Effects of the ether extract of *Bidenes aurea* on lesions induced by 30% NaCl (ulcer index, UI) and changes in mucosal sulfhydryl groups content (120 min).

0.014 and $3.168 \pm 0.107 \mu\text{mol/g}$ tissue respectively. Necrotizing agents caused a marked reduction in the mucosal non-protein SH contents ($p < 0.001$) in all experimental models (Figs. 1, 2 and 3). However, rats pretreated with ether extract increased significantly this fraction at all doses tested on absolute ethanol (250 mg kg^{-1} : 1.30 ± 0.31 $p < 0.05$;

125 mg kg^{-1} : 1.01 ± 0.12 $p < 0.01$ and 62.5 mg kg^{-1} : 0.65 ± 0.12 $p < 0.05$, Fig. 1) and 0.6 N HCl induced-injury (250 mg/kg : 1.39 ± 0.36 ; 125 mg kg^{-1} : 1.20 ± 0.09 and 62.5 mg kg^{-1} : 1.09 ± 0.18 , $p < 0.05$, Fig. 3). Using 30% NaCl, only the highest dose, 250 mg kg^{-1} produced a significant enhancement of this parameter (1.66 ± 0.31 $p < 0.05$, Fig. 2).

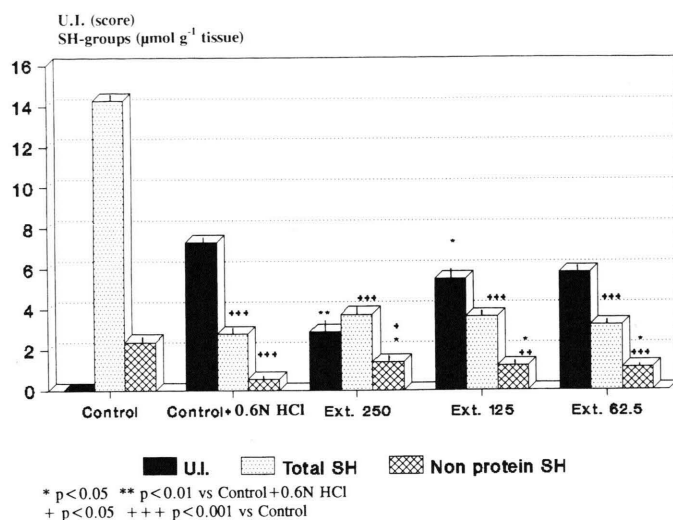


Fig. 3. Effects of the ether extract of *Bidens aurea* on lesions induced by 0.6 N HCl (ulcer index, UI) and changes in mucosal sulfhydryl groups content (120 min).

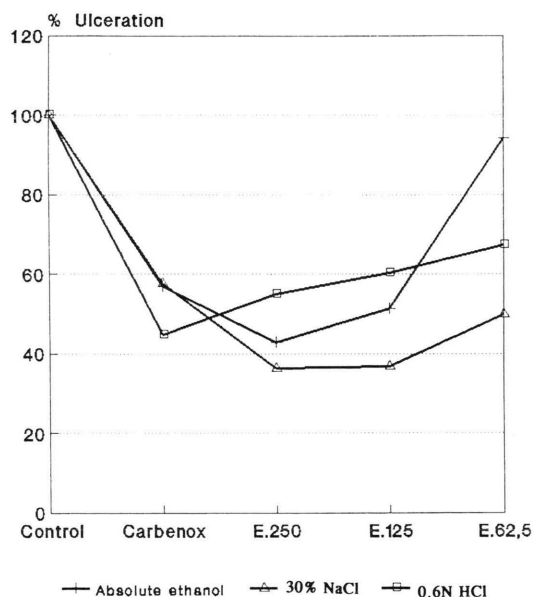


Fig. 4. Modifications produced by carbenoxolone (80 mg kg^{-1}) and the ether extract of *Bidens aurea* (250, 125 and 62.5 mg kg^{-1}) on ulcerations (%) induced by necrotizing agents on gastric mucosa.

Discussion

Exposure of the glandular mucosa of the rat stomach to high concentrations of necrotizing agents rapidly produces grossly evident focal hem-

orrhagic lesions. It has been suggested that these ulcers result from a decreased gastric mucus production (Wallace and Whittle, 1986), reduction in gastric glutathione content (Szabo, 1989) and decrease in gastric prostaglandin E_2 levels (Terano *et al.*, 1993). These agents induce gastric ischemia and increase the lipid-peroxide production in the gastric mucosal tissue indicating that oxygen-derived free radicals and lipid peroxidation are involved in the pathogenesis of these lesions (Salim, 1990). Thus, the increase of sulfhydryl compounds and removal of oxygen-derived free radicals, stimulate the healing of this class of acute gastric mucosal injury in the rat (Whittle, 1989).

The results of the present study show that the ether extract of *Bidens aurea* has a protective effect against injury in gastric mucosa induced by necrotizing agents. Best efficiency is against NaCl-induced lesions obtaining a significant decrease of damage with all doses tested (250, 125 and 62.5 mg kg^{-1}). In contrast, only the highest dose (250 mg kg^{-1}) was effective on 0.6 N HCl-induced gastric erosions. This dose produced an ulceration percent that fluctuated between 55.13% in rats treated with 0.6 N HCl to 36.25% on NaCl induced-ulcers.

This antiulcerogenic effect could not be only related to the characteristics of mucus gel since only

Table I. Effects of carbenoxolone and ether extract of *Bidens aurea* (120 min before ethanol) on gastric mucus secretion.

Treatment 120 min	Animals	Total mucus content [g]	Total proteins [mg/ml]	Hesoxamines [µg/ml]
Control + ethanol	6	0.55 ± 0.18	5.74 ± 0.60	66.97 ± 3.59
Carbenoxolone 80 mg/kg	6	1.02 ± 0.31 ***	11.03 ± 1.21 ***	80.01 ± 2.70 *
Ether extract 250 mg/kg	6	1.24 ± 0.09 ***	5.61 ± 0.77	139.62 ± 8.88 ***
Ether extract 125 mg/kg	6	0.31 ± 0.06	3.09 ± 1.75	69.54 ± 2.08
Ether extract 62.5 mg/kg	6	0.42 ± 0.10	5.39 ± 0.71	40.68 ± 3.16

* p < 0.005; *** p < 0.001 vs control.

Table II. Effects of carbenoxolone and ether extract of *Bidens aurea* (120 min before 30% NaCl) on gastric mucus secretion.

Treatment 120 min	Animals	Total mucus content [g]	Total proteins [mg/ml]	Hesoxamines [µg/ml]
Control + NaCl	6	0.57 ± 0.04	2.62 ± 0.18	48.20 ± 1.82
Carbenoxolone 80 mg/kg	6	0.61 ± 0.05	3.78 ± 0.59	55.05 ± 1.25
Ether extract 250 mg/kg	6	0.78 ± 0.24 *	6.75 ± 2.12 **	28.41 ± 6.04
Ether extract 125 mg/kg	6	0.75 ± 0.15	4.67 ± 0.95 *	19.34 ± 1.53
Ether extract 62.5 mg/kg	6	0.76 ± 0.12	3.12 ± 0.41	17.09 ± 1.21

* p < 0.05; ** p < 0.01 vs control.

Table III. Effects of carbenoxolone and ether extract of *Bidens aurea* (120 min before 0.6 N HCl) on gastric mucus secretion.

Treatment 120 min	Animals	Total mucus content [g]	Total proteins [mg/ml]	Hesoxamines [µg/ml]
Control + HCl	6	0.66 ± 0.07	5.61 ± 0.56	144.76 ± 24.22
Carbenoxolone 80 mg/kg	6	0.59 ± 0.37	4.92 ± 0.37	128.73 ± 13.31
Ether extract 250 mg/kg	6	0.61 ± 0.57	4.96 ± 0.65	156.25 ± 3.62 *
Ether extract 125 mg/kg	6	0.39 ± 0.21	3.18 ± 0.47	115.15 ± 9.79
Ether extract 62.5 mg/kg	6	0.44 ± 0.20	1.39 ± 0.24	146.53 ± 3.29

* p < 0.05 vs control.

the groups that received 250 mg kg⁻¹ of ether extract and after 1 ml of absolute ethanol or 30% NaCl increased significantly the amount of gastric mucus and its glycoprotein content. There are many reasons for assuming that the mucus layer alone is incapable of protecting the underlying mu-

cosa and that other defensive mechanisms must come into play, such as secretion of bicarbonate or restitution of damage microcirculation (Robert *et al.*, 1983). These mechanisms constitute the base of the gastric cytoprotection and are mediated by at least two different factors, one concerning pros-

taglandins (Terano *et al.*, 1993) and the other involving SH-containing compounds of the mucosa (Szabo, 1989).

Vasoprotection seem to be the common mechanism of gastroprotection by SH-related compounds because they decrease the chemically-induced vascular damage and increase the gastric mucosal blood flow (Glavin *et al.*, 1992). Moreover, the intracellular glutathione may play an essential role in the maintenance of gastric cellular integrity against ethanol injury (Mutoh *et al.*, 1991). Indeed, an augmentation in gastric non protein-SH content limits the production of oxygen-derived free radicals (Itoh and Guth, 1985).

The ether extract of *Bidens aurea* increased significantly the mucosal non protein-SH content at all doses tested on absolute ethanol and HCl-induced injury. Using 30% NaCl the highest dose (250 mg kg⁻¹) also produced an enhancement of this parameter.

In addition, numerous flavonoids exert significant scavenging properties on oxygen radicals *in vivo* and *in vitro* and this effects has been related with antiulcer activity (Martín *et al.*, 1994; Pérez-Guerrero *et al.*, 1994). In this way, Salim (1990) has demonstrated that oxyradicals scavengers could be beneficial in these experimental models.

Phytochemical analysis of *Bidens aurea* shows that the ether extract is especially rich in polyphenolic compounds mainly aurones and chalcones, from which the genin maritimetin and sulfuretin have been identified in this study. Several compounds of this nature showed antiulcer activity. Sasajima *et al.* (1978), found that sophoradine a isoprenyl chalcone present in the root of *Sophora subprostata*, exhibits an gastroprotective effect in both, Shay's pylorus ligated and restraint stress rats. Using the same experimental models, Kyogoku *et al.* (1979), showed the antiulcerogenic properties of more than 30 chalcones with a structure-activity relation ship. More recently, Hatayama *et al.* (1985) have demonstrated the same effect with three dihydrochalcone derivatives of sophoradin. It is possible that the gastroprotective effect of the ether extract of *B. aurea* on induced ulcers by necrotizing agents could be due partly to the presence of glycosides of aurones and chalcones and could be explained by a complex mechanism involving a slight increase in the mucus gel concentration and its glycoprotein content and the enhancement of non-protein sulfhydryl compounds. Probably the scavenger properties of its constituents are also involved, but this assumption requires further explorations.

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