Antiulcerogenicity of the Flavonoid Fraction from *Erica andevalensis* Cabezudo-Rivera

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Investigations were carried out to determine the antiulcerogenicity of the flavonoid fraction (ethyl acetate extract) of *Erica andevalensis* Cabezudo-Rivera on gastric ulceration induced by different experimental models. Oral treatment with the ethyl acetate extract and the major flavonoid (myricetin 3-*O*-D-galactoside) were found to be effective to prevent gastric ulceration induced by cold-restraint stress in rats . Statistically significant ulcer index values with respect to the control group were observed. Mucus content was not increased although it was accompanied by an increase in proteins and hexosamines. In pyloric-ligated animals flavonoids showed a significant reduction in the number and severity of the ulcers. Under the same conditions acidity did not decrease with the flavonic extract and myricetin 3-*O*-D-galactoside significantly as compared to control. Gastric ulcers induced by oral administration of absolute ethanol were reduced by pretreatment with the flavonoid extract of doses from 125 to 250 mg/kg and the isolated flavonoid of 25 mg/kg *p.o.* However neither the flavonic extract nor the isolated flavonoid induced changes in the amount and glycoprotein content of gastric mucus.

Introduction

Flavonoids have a broad scale of biological activities, among which their antiulcer effects are outstanding (Alarcón de la Lastra *et al.*, 1992, 1993; Gabor, 1986). Some of them have been shown to increase the mucus content of gastric mucosa, showing cytoprotective effects (Alarcón de la Lastra *et al.*, 1993; Motilva *et al.*, 1994).

Erica andevalensis Cabezudo-Rivera (Ericaceae) is a perennial plant widespread in the Andevalo (Huelva) which has been used in folk medicine as an urinary antiseptic. It has a high content of flavonoid substances (Aumente *et al.* , 1988).

The aim of this work was to evaluate the antiulcer efficiency of the flavonoid fraction of *Erica andevalensis* against different models of experimental gastric ulceration. In addition the present investigation was designed to examine gastric mucus content and composition, since gastric mucus is considered to be a physiological barrier which plays an important role in protecting gastric mucosa.

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Material and Methods

General procedure

Melting point was determined using a microscopy Thermovar HT 1 B11 and is incorrected. Ultraviolet absorption (UV) and infrared (IR) spectra were measured on a Perkin Elmer Lambda 3 spectrophotometer and a Perkin-Elmer 681 spectrophotometer, respectively. ^{1}H-RMN spectrum was recorded with a Bruker ACTF spectrometer at 200 MHz. EI-mass and FAB-mass spectra were obtained with a Kratos MS-80-RFA mass spectrometer at 70 eV. Analytical GC was carried out with a Hewlett Packard 5710A chromatograph equipped with a flame ionization detector, using a BP-1 fused silica glass capillary column (25 m x 0.53 mm) (temperature programmed from 100 to 240°C at 2°C min^{-1}). The temperatures of the injector and the detector were kept at 250°C and 300°C, respectively. Carrier gas was Helio and

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retention indices (Rt) were calculated according with hexoses as reference compounds. Silica gel 60 (Merck, 0.063–0.2mm) was used for CC and precoated silica gel plates (Merck, Kieselgel 60 F_{254}) was used for TLC.

Plant material

Flowering tops of *Erica andevalensis* were collected in the Andevalo region (Huelva, Spain) and were identified at the Laboratory of Botany of the Faculty of Pharmacy, University of Sevilla and deposited as a voucher specimen, labelled SEVF 117980. The samples were air-dried at room temperature, 22–24°C, and processed to a moderately coarse powder.

Extraction and isolation

The material (1 kg) was extracted successively in a Soxhlet first with chloroform and then with methanol; this extract was concentrated to dryness under vacuum and the residue was dissolved in boiling water. The aqueous solution was partitioned by the method of Netien and Lebreton (1964) to produce diethyl ether (40g) and ethyl acetate (71g) extracts. The ethyl acetate extract was chosen for gastric antiulcer studies. This extract was fractionated by column chromatography (120 x 4 cm) on silica gel (210g) with EtOAc-MeOH-H_2O (80:3:3) as an eluent to obtain one hundred and fifty six 20ml fractions. A pure flavonoid glycoside was isolated from the fractions No 58–87:

Myricetin 3-*O*-D-galactoside: Fine yellow cristals, m.p. 201°C, UV l_{max}: 256, 362 nm; IR: 3430, 1640, 1590, 1150 cm^{-1}; EIMS *m/z* (M^+-galactose) 318, FABMS *m/z* (M^++H) 481; ^{1}HNMR (MeOD), d (ppm), 5.22 (1H, d, *J*=8 Hz, H-1") 7.49 (2H, s, H-2′H-6′) 6.39 (1H, d, *J*=2Hz, H-8) 6.19 (1H, d, *J*=2 Hz, H-6).

In a separate experiment, 1.2 mg of the compound was dissolved in Me_2CO and distilled H_2O and R. Kiliani (0.5 ml) was added (HOOC-CH_3/H_2O/HCl, 3.5/5.5/1mL). The solution was heated at 100°C for 2h, extracted four times with EtOAc (20ml) and treated in the usual manner. TLC analysis of the organic extract permitted identification of myricetin by comparison with an authentic sample; in addition to a sugar identified as galactose by GC.

Animals

Male Wistar rats, 180–200 g, were placed in single cages with wire-net floors in a controlled room (temperature 22–24°C, humidity 70–75%) and were fed a normal laboratory diet. The animals were deprived of food for 24 h before experimentation but alloved free access to tap water throughout. All studies were carried out using 6–8 rats per group.

Drug preparation and treatment

Ethyl acetate extract (250, 125 and 62.5 mg/kg) and glycoside flavonoid (25 mg/kg) were suspended in distilled water. Omeprazole (1H-benzimidazole, 5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]) (Astra S.A., Barcelona, Spain) (10 mg/kg) and carbenoxolone (Olean-12-en-29-oic acid, 3-(3-carboxy-1-oxopropoxy)-11-oxo-,(3β,20β) (Leo S.A., Barcelona, Spain) (80 mg/kg) were used as standard drugs. The drugs were prepared freshly each time and administered by intragastric route. Control rats received distilled water in a comparable volume (1 ml/100g) by the same route.

Stress-induced gastric ulcer: inmobilization and cold

Ulceration was induced by immobilizing the rat in a cylindrical cage enclosed at its ends and maintained at 3–5°C during 3 hr (Senay and Levine, 1967). Different groups of animals were administered the ethyl acetate extract and omeprazol 1 hr before starting the experiment. Thereafter, the animals were killed and the stomachs were cut along the smaller curvature and washed carefully with 4 ml of physiological saline (Bulbena *et al*, 1986). The mucosa was scraped with a spatula to separate the gastric mucus, which was put aside for study. Any gastric lesions were observed immediately. On examination, the ulcers produced were measured and expressed in terms of the ulcer index (U.I.) in mm^2.

In addition, percentages of ulcerated (U.S.) and haemorrhagic stomachs (H.S.) were also expressed.

Protection against absolute ethanol

Ulceration was induced as described by Robert (1979) by instillation of 1 ml of the absolute etha-

nol . The different doses of the ethyl acetate extract and carbenoxolone were administered to different groups of animals one hour before the administration of the necrotizing agent. One hour after the experimental period, the animals were sacrificed using an overdose of anaesthetic and their stomachs removed and opened along the greater curvature. Any lesions were examined macroscopically. Length and width of the ulcers were measured and the product was expressed as the ulcer index (U.I.) in mm^2.

Biochemical study of the gastric mucus

The gastric mucus obtained by scraping the mucosa was homogenized in 4 ml of distilled water. The weight of mucus (g) was obtained from the difference between the weight of homogenate and the original 4 ml of water (Robert, 1979). Total proteins (mg/ml) were determined from one portion of the homogenate (1ml), following the colorimetric technique of Lowry *et al.* (1951). The hexosamine content (mg/ml) was determined from a second portion of the homogenate (2 ml). This was subjected firstly to acid hydrolysis with 1 N HCl, and then a colorimetric reaction in the presence of *p*-dimethyl-aminobenzaldehyde according to the method described by Boas (1953). In order to avoid variations in either the criteria of gastric mucosal lesion or scraping technique, they were performed by the same person.

Pylorus-ligated gastric secretion and ulceration

The pylorus-ligated rat model first described by Shay *et al.* (1954) was used. The drugs were administered orally 1 h before starting the experiments. The pylorus was tied under light ether anaesthesia. The cardia was ligated and the whole stomach was removed. The gastric content was collected and centrifuged to obtain a clear fluid. The total volume was measured. The stomach was cut open along the greater curvature and washed gently under running tap water. On examination, the ulcers produced were measured and expressed in terms of the U.I. (mm^2).

Estimation of acid and pepsin content

Samples of gastric content (1 ml) were analysed for hydrogen ion concentration by potentiometric titration with 0.01 M NaOH. The acid content was expressed as mEq/l. The colorimetric method of Lowry (1951) was used to determine the total protein in samples expressed as mg pepsin (ml of gastric content).

Statistical analysis

Values are given as arithmetic means ± S.E.M. The significance of differences between means was evaluated by Student's t-test for unpaired data. For gastric mucosal lesions the Mann-Whitney U-test was used.

Results

The results obtained in the ulcer induced by restraint and cold indicate that the ethyl acetate fraction of the flavonoid extract (250, 125 and 62.5 mg/kg) and the glycoside isolate from it, myricetin-3-galactoside (25 mg/kg) gave a high level of gastric protection. The same range was obtained with 10 mg/kg of omeprazole (Table I).

In relation to the amount of gastric mucus (Table II) it was not significant compared to control. In contrast the concentration in proteins and hexosamines with the extract and the flavonoid compound increased significant as compared to control (Table I).

In 6h-pyloric ligated animals, the ethyl acetate extract showed a significant reduction in the number and severity of ulcers and UI decreased dose-dependently although the results were lower than those obtained with omeprazole (Table II). Under the same conditions pepsin concentration decreased but acidity did not decrease significantly as compared to control. In addition, gastric content was significantly increased as compared to control with 250 and 125 mg/kg of the extract. Similar values were obtained with the myricetin 3-*O*-D-galactoside.

Pretreatment with the ethyl acetate extract 250 and 125 mg/kg and myricetin 3-*O*-D-galactoside 1h before administration of absolute ethanol to rats significantly prevented the formation of gastric lesions (Table III). In relation to the quantity and quality of mucus secretion, gastric mucus and hexosamine contents showed similar values as compared with control groups while the total protein output was increased significantly (Table III).

Table I: Effects of ethyl acetate extract, myricetin 3-*O*-D-galactoside and omeprazole on ulcer formation and glycoprotein contents in cold-restraint-induced gastric ulcer in rats.

Treatment	Animals	%UE	%HE	UI [mm^2]	Total proteins [mg/ml]	Hexosamines [µg/ml]
Control	6	100	70	39.16±0.62	0.43±0.03	20.87±2.76
Omeprazole	6	50	0	0.28±0.01	0.21±0.06	23.00±2.50
10 mg/kg				++	n.s	n.s.
EtOAc extract	6	100	45	6.50±1.24	4.52±0.07	96.50±9.13
62.5 mg/kg				++	***	**
EtOAc extract	6	100	47.5	6.33±1.27	4.95±0.07	102.56±1.15
125 mg/kg				++	***	***
EtOAc extract	6	100	50	0.45±0.42	4.10±0.01	119.57±7.79
250 mg/kg				++	***	***
Myricetin 3-O-D-galactoside	6	60	0	2.42±0.01	2.34±0.03	47.82±1.57
25 mg/kg				++	***	**

"U"-Mann Whitney test: ++ $p<0.01$; "t"-Student test: ** $p < 0.005$; *** $p < 0.001$; n.s. not significant; %UE: Ulcerated stomachs; HE: Haemorrhagic stomachs; UI: Ulcer index.

Table II: Effects of ethyl acetate extract, myricetin 3-*O*-D-galactoside and omeprazole on acidity [mEq/l], pepsin [mg/ml], gastric content [ml] and ulceration in pylorus-ligated rats.

Treatment	Animals	%UE	UI [mm^2]	Gastric content [ml]	Acidity [mEq/l]	Pepsin [mg/ml]
Control	6	100	50.87±2.01	5.58±0.55	44.20±0.75	29.70±2.30
Omeprazole	6	50	0.66±0.40	5.55±0.20	21.80±0.35	15.00±0.90
10 mg/kg			++	n.s.	*	*
EtOAc extract	6	100	13.87±0.81	6.10±0.09	38.10±0.20	11.94±1.22
62.5 mg/kg			+	n.s.	n.s.	*
EtOAc extract	6	83.3	5.25±1.60	6.91±0.13	39.10±0.73	13.08±0.45
125 mg/kg			++	*	n.s.	*
EtOAc extract	6	66.6	2.04±0.7	7.56±0.52	29.01±0.48	14.57±0.72
250 mg/kg			++	*	n.s.	*
Myricetin 3-O-D-galactoside	6	100	4.20±1.25	7.62±0.47	30.94±0.92	15.59±1.11
25 mg/kg			++	*	n.s.	*

"U"-Mann Whitney test: + $p<0.05$; ++ $p<0.01$; "t"-Student test: * $p<0.05$; n.s. not significant; %UE: Ulcerated stomachs; UI: Ulcer index.

Table III: Effects of ethyl acetate extract, myricetin 3-O-D-galactoside and carbenoxolone on ethanol induced gastric mucus secretion and ulceration in rats.

Treatment	Animals	UI [mm^2]	Mucus content [g]	Total proteins [mg/ml]	Hexosamines [µg/ml]
Control	10	305.60±6.70	0.55±0.01	5.74±0.6	66.97±3.50
Carbenoxolone	10	52.77±1.30	1.02±0.03	11.03±1.2	73.06±5.42
80 mg/kg		++	**	**	**
EtOAc extract	6	198.98±2.08	0.57±0.09	7.86±0.53	59.04±4.09
62.5 mg/kg		n.s.	n.s.	*	n.s.
EtOAc extract	6	112.48±2.08	0.51±1.40	7.38±0.42	60.02±4.13
125 mg/kg		+	n.s.	*	n.s.
EtOAc extract	6	95.40±1.67	0.45±0.03	7.93±0.31	63.76±3.21
250 mg/kg		+	n.s.	*	n.s.
Myricetin 3-O-D-galactoside	6	87.20±19.02	0.30±0.09	4.75±0.70	75.33±4.04
25 mg/kg		+	n.s.	n.s.	n.s.

"U"-Mann Whitney test: + $p<0.05$; ++ $p<0.01$; n.s. not significant; t"-Student test: * $p<0.05$; ** $p<0.005$; n.s. not significant; UI: Ulcer index.

The flavonoid glycoside, myricetin 3-*O*-D-galactoside, was isolated from the ethyl acetate extract of *E. andevalensis*. The EI-mass spectrum of compound showed the molecular ion at *m/z* 318 while the FAB-mass spectrum showed a peak at *m/z* 481 (M^++H). The IR spectrum of flavonoid exhibited absortion bands due to hydroxyl (3430 cm^{-1}), an a,b-unsaturated ketone (1650 cm^{-1}). The UV spectrum of isolated compound showed absorption maxima at 256 and 362 nm. The 5–7-disubstitution in ring A was evident by the presence of two meta coupled protons at d 6.19 (*J*= 2Hz) and 6.39 (*J*= 2Hz) for one proton each, ascribed to H-6 and H-8 respectively. The ^{1}H-RMN spectrum also showed one singlet at d 7.49 assigned for two protons H-6¢ and H-2¢. The position of the C-1 proton of galactose at d 5.22 (*J*= 8Hz) indicated that the 3 position of the aglycone was occupied by galactose. This is further supported by the acidic hydrolysis of compound. Acidic hydrolysis of it afforded a sugar and a aglycone which was identified as myricetin by the comparison with an authentic sample; in addition the sugar was identified as galactose by GC; therefore it was characterized as myricetin 3-*O*-D-galactoside.

Discussion

The results of the present study in rats clearly show that the ethyl acetate extract and major flavonoid isolated from *Erica andevalensis* have been effective in various types of experimentally induced gastric ulcers. Oral treatment with 62.5, 125 and 250 mg/kg of the flavonic fraction dose-dependently and 25 mg/kg myricetin 3-*O*-D-galactoside reduced the incidence of gastric mucosal damage in pylorus-ligated rats, although it does not decreased either acidity or pepsin secretion with the tested doses. These results suggest that the antiulcerogenic effects of them are independent of the inhibition of acid secretion.

The ethyl acetate extract and myricetin 3-*O*-D-galactoside have also been found to be effective in reducing in a highly significant way, the ulceration induced by cold-restraint stress. This was accompanied by a significative increase in the quality of the mucus. The etiology of acute gastric stress erosions is unknown but current evidence supports a multifactorial etiology (Cho *et al.*, 1992). The weight of evidence suggests failure of cytoprotective mechanisms rather than increased acid secretion. Back diffusion of acid appears to be a consequence rather than a cause of acute stress ulcers (Kivilvoto *et al.*, 1988).

Gastric mucus glycoproteins are believed to play an important role in the defensive mechanisms against gastric ulceration. The mucus layer acts as an unstirred mucus-bicarbonate mucosal barrier to acid and pepsin digestion (Allen *et al.*, 1993). In conditions of emotional tension, there is not only a greater destruction of mucus and a lesser synthesis of its components but also a quality change, affecting the translation, acylation and glycosylation of the ribosomal peptides (Tsukada *et al.*, 1989). In this context, some flavonoids increase its glycoprotein content (Alarcón de la Lastra *et al.*, 1993; Motilva *et al.*, 1994).

In addition, most investigators implicate mucosal ischemial as a major causative factor of stress ulceration. The focal nature of the ischemic areas may indicate local release of vasoactive and cytotoxic mediators among which oxygen-derived free radicals are likely candidates as are leucotrienes and PAF (Jacobson, 1992). Flavonoids exert significant scavenging properties on oxygen radicals *in vivo* and *in vitro* and can interfere with the production of arachidonic acid metabolites thorough lipoxygenase or cyclo-oxygenase pathway (Chen, 1990). Active oxygen species such as superoxide anion and its interconversion to hydroxyl radical attack unsaturated membranes lipids with release of intracellular components e.g. lysosomal enzymes and induction of lipid peroxidation that causes further tissue damage. So it is possible that enhancing mucus quality and scavenging oxygen derived free radicals would contribute to the antiulcerogenicity or *Erica andevalensis* in this experimental model.

Pretreatment with 125 and 250 mg/kg of the flavonoid extract and 25 mg/kg of myricetin 3-*O*-D-galactoside considerably protect the gastric mucosa from the lesions induced by absolute ethanol, however mucus and hexosamine content are not significantly enhanced. These results lead us to reject the protagonism role of gastric mucus in the protective effect in this model.

It has been reported that in <3 minutes after a necrotizing insult, two circulatory responses become prominent, suggesting that the mucosal microvasculature may have singled out as a prime

target in chemical injury. These responses include (a) vasospasm of venules and dilation of arterioles, reflecting vascular congestion and slowing or stasis of blood flow and (b) increased capillary permeability to the macromolecules. The consequences of these circulatory responses are ischemic hypoxia and edema, (Jacobson, 1992). Ischemia is known to incite inflammatory changes in gastrointestinal mucosa. Recent report has shown antiphlogistic properties for the 3-*O*-β-D-glucuronide of myricetin. This principle exhibited a very strong anti-inflammatory effect on carrageenan-induced edema in the rat hind paw (Hiermann *et al.*, 1991).

A singular characteristic of the inflammatory process is the enhanced vascular permeability (Glavin and Szabo, 1992). In this context, previous experiments showed a significant inhibition of the enhanced vascular permeability caused by histamine in animals treated with the ethyl acetate extract (Ayuso *et al.*, 1993). In addition, endogenous mediators, such us lipid peroxidation products, vasoactive amines or peptides as well as oxygen free radicals are supposed to be involved (Jacobson, 1992).

In summary, data presented here confirm that the ethyl-acetate extract of *Erica andevalensis* exerts gastroprotective activity which could be partly explained through antioxidant and vascular mechanisms. It is probably that the myricetin-3-galactoside isolated from extract could be implicate in these mechanisms. Preepitelial mechanisms as the enhancement of physicochemical properties of mucus gel could also be involved.

The flavonoid glycoside, myricetin 3-*O*-D-galactoside, was characterized by direct comparison (UV, IR, ^{1}H-RMN, EI-MS, FAB-MS, m.p.) with published data (Kagan, 1967). Information in detail on the work-up procedure and copies of the original spectra are obtainable from the author of correspondence.

In this study it has been reported for the first time, the isolation of myricetin 3-*O*-D-galactoside and the antiulcer activity recorded from *Erica* species.

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