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Chemical Characterization and Biological Effects of Sicilian *Opuntia ficus indica* (L.) Mill. Fruit Juice: Antioxidant and Antiulcerogenic Activity

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The juice of whole fruits of Sicilian cultivars of prickly pear (*Opuntia ficus indica* (L.) Mill.) was investigated, and the contents of ascorbic acid, total polyphenols, and flavonoids were determined. In the juice, ferulic acid was the chief derivative of hydroxycinnamic acid and the mean concentration of total phenolic compounds was 746 μg/mL. The flavonoid fraction, analyzed by high-performance liquid chromatography−diode array detection, consisted of rutin and isorhamnetin derivatives. The juice showed antioxidant activity in the DPPH• test, probably due to the phenolic compounds that are effective radical scavengers. The preventive administration of the juice inhibited the ulcerogenic activity of ethanol in rat. Light microscopy observations showed an increase in mucus production and the restoration of the normal mucosal architecture. The juice is nutritionally interesting, and its dietary intake could provide protection against oxidative damage.

KEYWORDS: ***Opuntia ficus indica*; fruit juice; antioxidant; flavonols; antiulcer activity; mucosa structural changes**

INTRODUCTION

Opuntia ficus indica (L.) Mill., a plant that could be considered as a symbol of Sicily, grows in all of the semiarid countries throughout the world and is especially cultivated in the Mediterranean area and in Central America. The plant is mainly cultivated for its fruit (prickly pear), although in some countries different parts of the plant are utilized in the food and cosmetic industry (*1*−*4*).

The fruit is juicy and sweet, with a thorny peel and a large number of small and hard seeds. In recent years, many countries have increased the production of prickly pears, whose fruit is nutritionally interesting; in fact, it is rich in sugars, vitamin C, pigments, and minerals such as Ca, Na, Mg, Zn, Fe, and according to some authors, also Mn and Se (*5*−*8*). For centuries, this fruit has been generally consumed in the same country where it was produced, but nowadays, probably as a consequence of the emigrations, the fruit is widely commercialized in North Europe and in North America. Because the long-term storage of the fruits can be problematic (*9*), in many countries, studies on the processing and storage of its juice are in progress; moreover, the juice can be either consumed as such or employed in the food industry in the preparation of beverages with improved taste and/or nutritional properties.

Within a project on *O. ficus indica* (L.) Mill. funded by Regione Siciliana, we have investigated a number of biological activities of prickly pear juice. We here report the results of the study of the antiulcer activity of *O. ficus indica* fruit juice on ethanol-induced ulcer in rat.

There are no scientific papers in the literature about the presence in this fruit of phenolic compounds, which could have an important effect on the oxidative stability and microbiological safety of the juice. Polyphenols, and especially flavonoids, are important because of their contribution to the sensory quality of fruits and also because of their nutritional and biological properties (*10*). Therefore, we also report the antioxidant properties, the total phenolic content, and the flavonoid composition of *O. ficus indica* fruit juice.

MATERIALS AND METHODS

Plant Material. *O. ficus indica* fruits (50 kg) were manually collected in a cultivation located in San Cono (CT, Sicily). The whole fruits (95% yellow cultivar and 5% red cultivar) were washed and ground by a Musermax double-bladed mill. They were then pressed at 3 atm in a hydraulic press, maintaining the material under pressure for 6 h. A cloudy juice was obtained, while solids remained in press. The cloudy juice was filtered through inox net, and small aliquots (25 mL)

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were stored at −18 °C. Samples of 25 mL were defrosted immediately before the experiments. We determined vitamin C, total polyphenols, and flavonoids of the whole juice.

Samples of 25 mL were concentrated to 5 mL by a Rotavapor (35 °C) and extracted with 10 mL of diethyl ether (two times); an organic fraction and an aqueous fraction were obtained. The aqueous fraction was extracted with 10 mL of ethyl acetate (two times); an organic fraction and an aqueous fraction (fraction I) were obtained. The organic fractions were pooled (fraction II) (*10*). At the end of the process, both fractions (I and II) were evaporated to dryness and redissolved in 2 mL of methanol. Both fractions were subjected to high-performance liquid chromatography (HPLC) analysis in order to evaluate flavonoids soluble in water or in organic solvent.

Determination of Total Phenolics. The phenol content was measured by the Folin-Ciocalteau reagent (*11*) using gallic acid as standard. One milliliter of the whole juice was mixed with 5 mL of Folin-Ciocalteau reagent (previously diluted 10-fold with distilled water) and 15 mL of sodium bicarbonate (20 g/100 mL), and the mixture was diluted to 100 mL with distilled water. The solution was kept in the dark at room temperature for 2 h; the absorbance was then measured at 760 nm with a model UV-2401 PC spectrophotometer (Shimadzu, Milano, Italia). All reagents were purchased from Sigma-Aldrich. The mean value of total phenolics content was obtained from triplicate experiments.

Determination of Flavonoids. One milliliter of the whole juice was diluted to 5 mL of dimethylformamide and analyzed by HPLC for the determination of the flavonoid profile. Fraction I and fraction II were analyzed as such, also by HPLC. Isorhamnetin was determined after acid hydrolysis of the flavonoid glycosides at 90 °C for 1 h with 1.2 M HCl in aqueous methanol (1:1 v/v) and HPLC analysis.

HPLC-DAD Analysis. A Shimadzu HPLC apparatus, equipped with two LC-10AD-Vp pumps, a SCL-10A-Vp system controller, a GT-154 on line degassing device, a Rheodyne (Perugia, Italia) injector with 20 μL loop, a BER 1000 P oven, and a SPD-M10A Vp diode array detector was used. A 250 mm × 4.6 mm i.d., 5 μm Alltime ODS column, fitted with a 7.5 mm × 4.6 mm i.d., 5μm, Alltime guard column (Alltech, Milano, Italia) was used. The injection volume was 20 mL. The mobile phase consisted of two eluents: (W) water and (A) acetonitrile, adjusted to pH 3.1 with acetic acid. The flow rate was set at 1.0 mL/min, and the gradient was as follows: initial, 95% W and 5% A; 10 min linear change to 75% W and 25% A; 10 min linear to 60% W and 40% A; 10 min linear to 50% W and 50% A; 5 min linear to 0% W and 100% A maintained for 5 min; 10 min to 95% W and 5% A maintained for 10 min for reequilibration at initial eluent composition before a new injection. The column temperature was set at 30 °C. Detection was at 365, 320, and 254 nm (4 nm bandwidth), and spectra were recorded between 220 and 400 nm at the apex of each peak.

Pure flavonoid standards (rutin, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside) and ferulic acid were purchased from Extrasynthese (Genay, France). Flavonoids in the sample were identified by comparison of retention times and spectra for each peak with the corresponding flavonoid standard. The total flavonol glycosides content was expressed as isorhamnetin-3-rutinoside equivalents, by an external standard method. The mean value was obtained from triplicate experiments.

HPLC-Atmospheric Pressure Chemical Ionization (APCI)-MS Analysis. The system used for the HPLC-MS analysis was a Shimadzu liquid chromatograph mass spectrometer LCMS-2010; an APCI interface was used in the negative ion mode. The HPLC apparatus was a Shimadzu system equipped with two LC-10AD-Vp pumps, a SCL-10A-Vp system controller, and a Rheodyne injector with 5 μL loop. A 150 mm × 2.1 mm i.d., 5 μm, Discovery C-18 column (Supelco, Milano, Italia) was used. The mobile phase consisted of two eluents: (W) water and (A) acetonitrile, adjusted to pH 3.0 with formic acid. The flow rate was set at 0.2 mL/min, and the gradient was as follows: initial 95% W and 5% A, maintained for 20 min; 30 min linear change to 60% W and 40% A; 5 min linear to 5% W and 95% A and then maintained for 5 min; 10 min to 95% W and 5% A and then maintained for 10 min for reequilibration at initial conditions. The APCI parameters were set as follows: probe voltage (kV), −4.00; probe temperature, 400 °C; block temperature, 200 °C; CDL temperature, 200 °C; Q-array voltage, −20 and −80 V; gas flow, 2.5 L/min.

Determination of Ascorbic Acid Content. The ascorbic acid content of the whole juice was determined by the AOAC Official Method of Analysis (*12*). The titration was performed in triplicate; the ascorbic acid amount was the mean value of three determinations.

Free Radical Scavenging Activity. Radical scavenging activity was assayed according to the method of Ohinishi et al. (*13*). An ethanol 1,1-diphenyl-2-picrylhydrazyl (DPPH$^\bullet$) solution (0.01 mM) was mixed with different concentrations of the juice, fraction I, and fraction II; the optical density change at 517 nm was measured 10 min later with a model UV-1601 spectrophotometer (Shimadzu).

The scavenging activity was measured as the decrease in absorbance of samples vs DPPH$^\bullet$ standard solution. The mean value was obtained from triplicate experiments. Results were expressed as percentage activity, and mean inhibiting concentrations (IC_{50}) were calculated by using the Lichfield and Wilcoxon test (*14*).

Antiulcer Activity. *Animals.* Male Wistar rats, weighing 180−200 g, kept in a controlled environment (temperature 22 ± 2 °C; humidity 60 ± 4%, natural light), maintained on a standard diet (S. Morini Mill rat GLP) and water ad libitum, were used. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D. M. 116192), as well as with the EEC regulations (O. J. of E. C. L 358/1 12/18/1986). Before the experiment, all of the animals fasted for 24 h, with free access to water.

Treatment. The rats were divided into three groups of 10 animals each. The first group (control) was treated, in the morning by gavage, with water (3 mL/rat/day) for 9 days. The second group of rats was treated, in the morning by gavage, with *O. ficus indica* fruit juice (3 mL/rat/day) for 9 days. On the ninth day, the third group received orally sucralfate (Sucral, Bioprogress) suspended in water (3 mL/rat) at the dose of 100 mg/kg, as reference drug. On the ninth day, after 60 min from the treatment, all of the animals received by gavage 90% ethanol at the dose of 0.5 mL/rat (*15*). After 60 min, all of the animals were sacrificed under ether anaesthesia. Their stomachs were cut along the greater curvature and washed with saline solution so as not to remove the mucus layer from the mucosa surface.

Macroscopic Observation. The lengths of each ulcer were measured and scored on an arbitrary scale (*16*). The ulcer index (UI) of each stomach was the sum of its scores. The UI is reported as arithmetic means of 10 values ± standard error. The significance of differences between means was evaluated by Student's *t*-test for unpaired data.

Microscopic Observation: Histology. The stomachs were extended on a cork surface to avoid deformities, and small pieces of every stomach were cut and fixed in neutralized 4% paraformaldehyde in 0.2% phosphate buffer for 4 h at 4 °C. The samples were washed with the same buffer and dehydrated in graded ethanols (30−100 °C) and, finally, embedded in bioplast (Biooptica, Milano, Italia). Sections (5 μm), obtained by a rotative microtome, were stained with periodic acid-Schiff. The reactive produces a characteristic carmine color with mucopolysaccharides, which constitute the gastric mucus (*17*). All samples were observed and photographed with BH_2 Olympus microscopy.

RESULTS

Total Phenolics and Flavonoids. The flavonoid profile of *O. ficus indica* whole fruit juice is shown in **Figure 1**. The main flavonoids identified in the present study were flavonol glycosides and, in particular, isorhamnetin glycosides (**Figure 2**). The total flavonol glycosides content (652.5 ± 38 μg/mL) was determined by HPLC by adding the amounts of the flavonol glycosides identified and the amounts of those peaks, which, on the basis of their retention times and UV spectra, we considered as flavonol glycosides. Isorhamnetin-3-rutinoside (54.6 ± 2 μg/mL) was quantified; small amounts of isorhamnetin-3-glucoside, rutin, and kaempferol-3-rutinoside were detected. Further studies are under way in order to determine also the identity of the other flavonol glycosides detected but

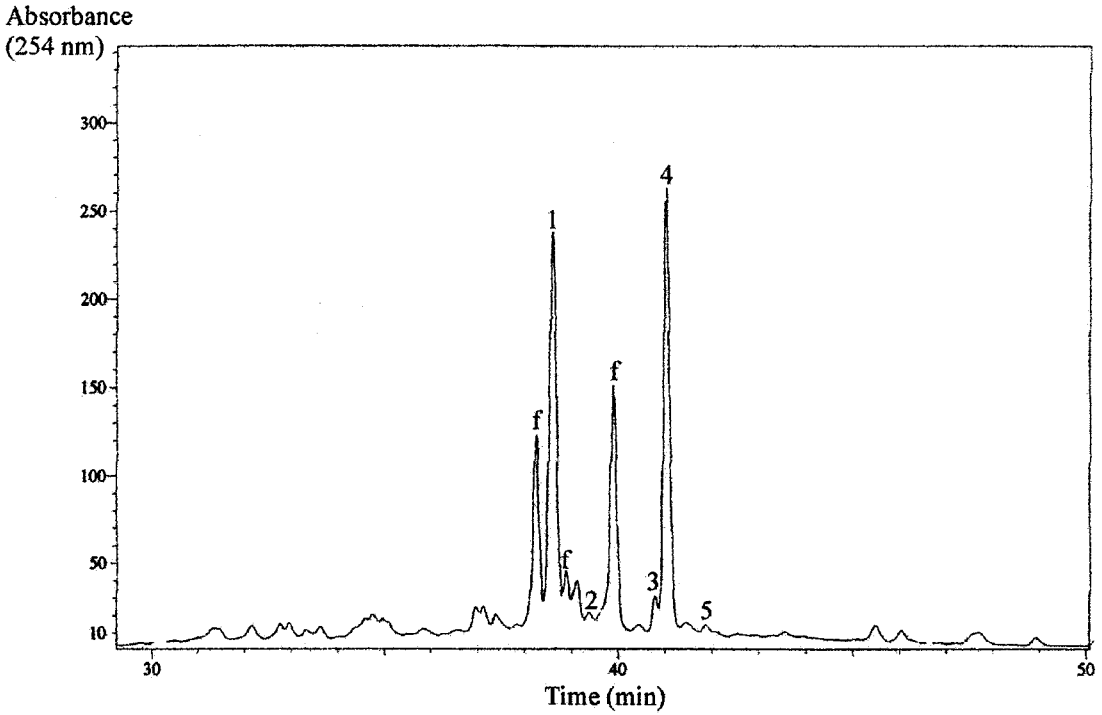

Figure 1. HPLC profile at 254 nm of *O. ficus indica* whole fruit juice. (1) Probably isorhamnetin triglycoside (retention time, 38.61 min); (2) rutin; (3) kaempferol-3-*O*-rutinoside; (4) isorhamnetin-3-*O*-rutinoside (retention time, 41.02 min); (5) isorhamnetin-3-*O*-glucoside; and (f) flavonol glycoside.

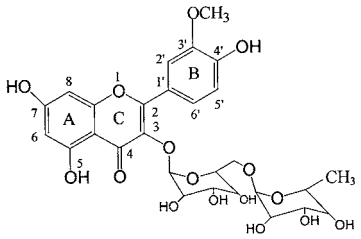

Figure 2. Structure of isorhamnetin-3-*O*-β-rutinoside.

not yet identified. On the basis of its retention time, UV spectra, and a preliminary HPLC-APCI-Neg-MS investigation of the juice, it seems very probable that peak 1 could be an isorhamnetin triglycoside. In fact, from the MS results (**Figure 3A**), it appears that the compound with a retention time of 38.61 min (see peak 1 of **Figure 1**) has an [M − H] ion at 769 mass units.

The fragmentation of the [M − H] ion of the triglycoside shows an ion at 623 mass units, which indicates a loss of a 146 mass unit indicative of a deoxyhexose sugar, and also exhibits an ion of *m*/*z* 315 suggesting a further loss of a deoxyhexose-hexose. The *m*/*z* 315 ion is indicative of the base aglycone component, isorhamnetin.

The presence of isorhamnetin-3-*O*-β-rutinoside was also confirmed by HPLC-APCI-Neg-MS. **Figure 3B**,**C** shows, respectively, the extracted ion chromatogram of *m*/*z* 623 and the corresponding MS spectra indicating the [M − H] ion of isorhamnetin-3-*O*-rutinoside (*m*/*z* 623) and the ion [M − 308] at *m*/*z* 315 corresponding to isorhamnetin. To confirm the presence of isorhamnetin derivatives as the main family of flavonol glycosides in the juice, an HPLC analysis after acid hydrolysis of the corresponding glycosides was carried out; this showed the aglycone isorhamnetin as the main peak. Therefore, isorhamnetin derivatives seem to characterize prickly pear juice, and this result could also be used for detecting adulterations. Fraction I (aqueous fraction) and fraction II (organic fraction) were also analyzed by HPLC-DAD, and the results showed that only fraction I had the same flavonoid profile as the juice itself, because the glycosides are mainly water soluble. Moreover, with the aim of assessing the general polyphenolic profile of *O. ficus indica* juice, we also carried out HPLC-DAD analysis for detecting other classes of polyphenolic compounds and preliminary evidence seems to indicate the presence of ferulic acid as the main representative of the hydroxycinnamic acids; although present in small amounts, it could contribute to the total antioxidant potential of the juice. The mean concentration of the total phenolic compounds in prickly pear juice is 746 ± 16 μg/mL as determined by the Folin-Ciocalteau method.

Determination of Ascorbic Acid Content. The ascorbic acid amount determined in *O. ficus indica* fruit juice was 26.9 ± 4.8×10^{-3} mg/100 mL.

Free Radical Scavenging Activity. *O. ficus indica* fruit juice and fraction I produce a 50% decrease vs the absorbance of DPPH$^{\bullet}$ standard solution at a volume of 6.75 and 7.68 μL, respectively (IC_{50}); fraction II, in contrast, failed to show any radical scavenging activity.

Macroscopic Observations of Gastric Mucosa. In the control rats that received only ethanol, intense and widespread gastric hyperaemia, thickened lesions, and many filiform lesions were evident; the UI value was 8.74 ± 1.5. The chronic pretreatment (9 days) with *O. ficus indica* fruit juice revealed a protective action against ethanol-induced ulcer. The stomachs showed an aspect close to normality; in fact, a significant reduction in gastric hyperaemia and in both number and severity of the lesions was observed. The UI significantly decreased to 1.53 ± 1.5 ($P < 0.05$ with respect to the control), nearly reaching the UI value of rats treated with sucralfate (1.28 ± 1.2) (**Table 1**).

Microscopic Observations: Histology. The gastric mucosa of rats treated for 9 days with water and then with ethanol (group I, control rats) showed necrotic lesions typically produced by the ulcerogenic agent. The surface epithelial cells were disrupted and desquamated. Several glandular bodies showed a restricted lumen. The interglandular spaces were dilated. Moreover, in the nonlesional areas, an increase in mucus production as response to the damage was evident (**Figure 4A**). The gastric mucosa of animals treated for 9 days with *O. ficus indica* fruit

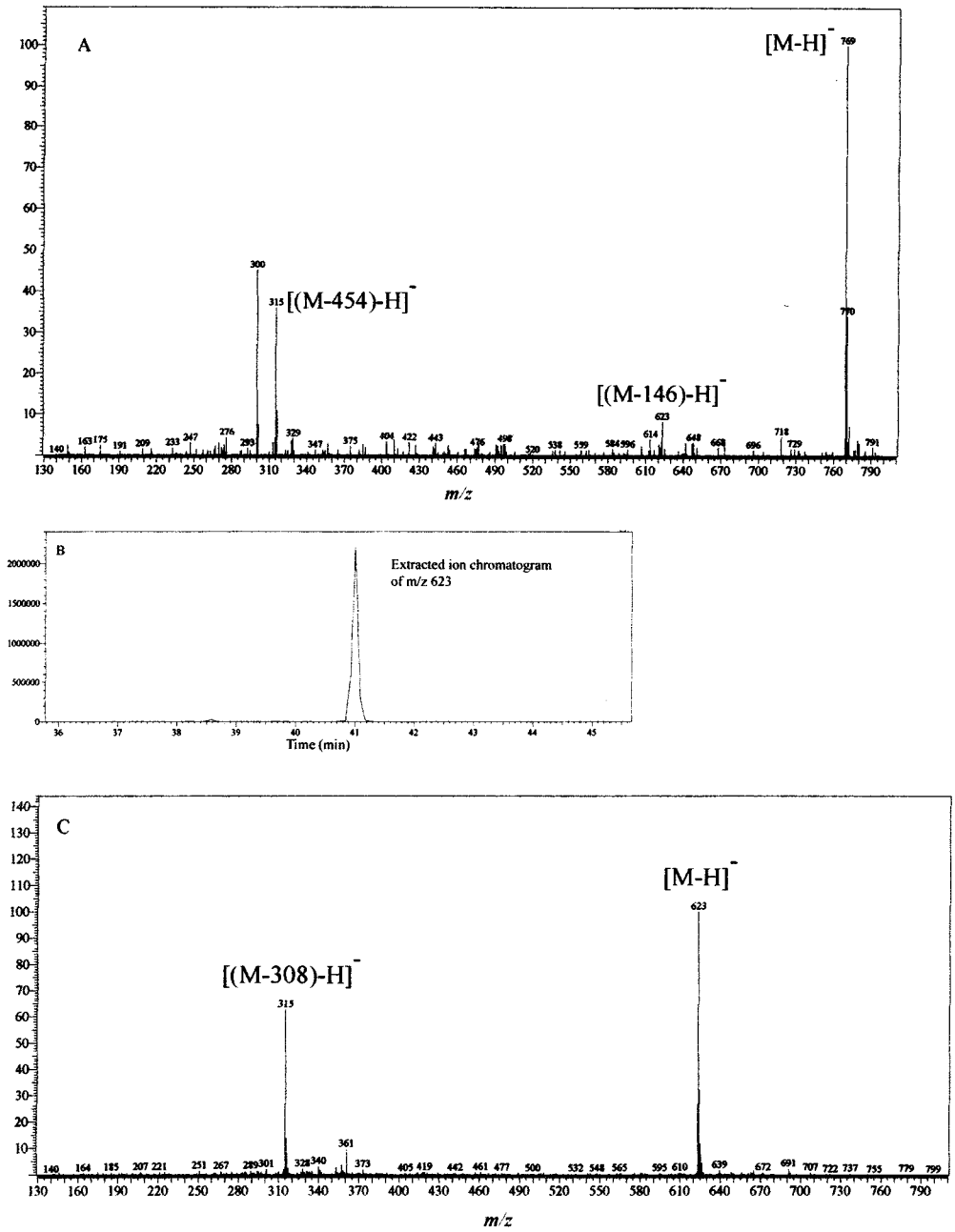

Figure 3. Preliminary HPLC-MS analysis of *O. ficus indica* fruit juice. (**A**) APCI-MS spectra of probable isorhamnetin triglycoside. (**B**) MS chromatogram of extracted (*m*/*z* 623) isorhamnetin-3-*O*-rutinoside. (**C**) APCI-MS spectra of isorhamnetin-3-*O*-rutinoside.

Table 1. Protective Effect of *O. ficus indica* Fruit Juice on Ethanol-Induced Ulcer in Rat; Evaluation of UI[a]

treatment	dose	UI ($\bar{X} \pm$ SE)
water	3 mL/rat	8.74 ± 1.5
O. ficus indica (L.) Mill. fruit juice	3 mL/rat	1.53* ± 1.5
sucralfate	100 mg/kg	1.28* ± 1.2

[a] Results are expressed as the mean of 10 animals ± standard error (SE). *P < 0.05 with respect to the control group.

juice and then with the ulcerogenic agent (group II) was almost normal in appearance; features appeared similar to those observed in the normal mucosa. In the "lamina propria" of gastric mucosa, fibroblasts were visible near the glandular portion. Intracellular mucus was localized inside the glandular pits only. The gastric glands recovered the normal dimensions, and the glandular spaces were reduced. The glandular bodies appear tubular, with a straight course and with funds lined up (**Figure 4B**). The gastric mucosa of animals treated with sucralfate (group III) appeared intact and showed a layer of mucus in the glandular pits and also in the neck cells (data no shown).

DISCUSSION

Several authors have studied the chemical composition of *O. ficus indica* juice and fruit (*8*, *18*, *19*), but the microconstituents amount revealed in the juice depends not only on the method employed for the processing of the juice but also on the degree of ripening, the storage temperature of the fruit, the cultivar, and the place of origin (*20*−*26*).

In Sicily, *O. ficus indica* grows in lavic lands, rich in metals that can influence the features of the plant and of the fruit. Moreover, in our experimental conditions, the juice was obtained by submitting the whole fruit to cold pressure, immediately after the harvesting. This process permitted not only short processing

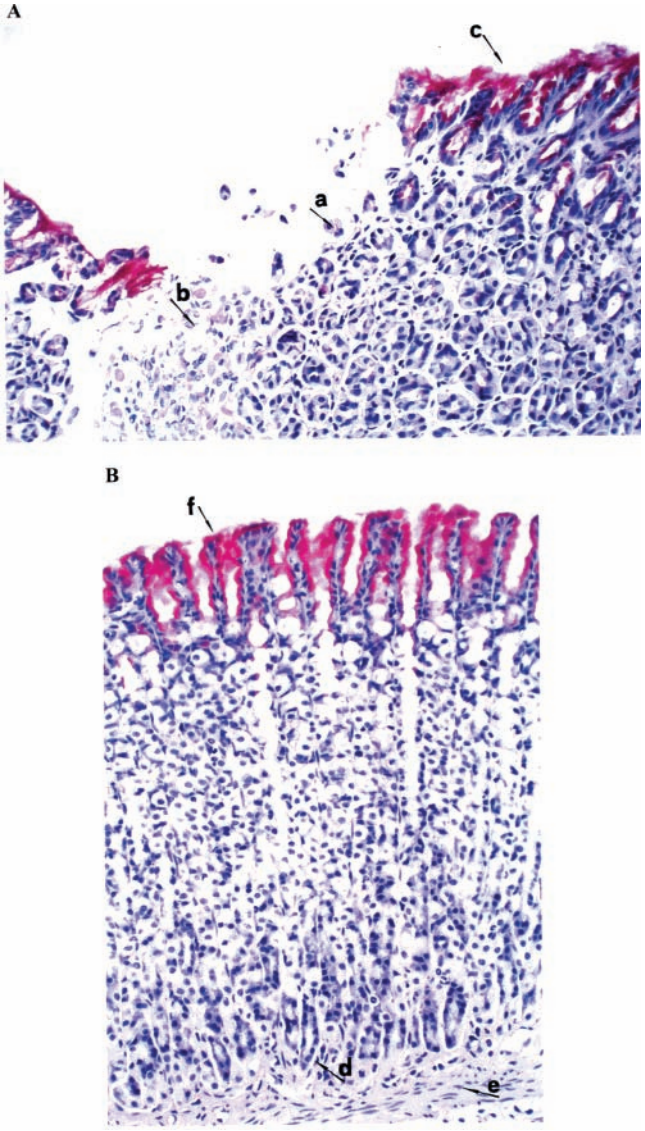

Figure 4. Light microscopy observations of rat mucosa gastric sections (magnification 20×). (**A**) Control rats: mucosa shows erosion of the surface epithelial cells (a), disorganization of deeper glandular structure (b), and increase in mucus production as response to damage (c). (**B**) *O. ficus indica* fruit juice-treated rats: mucosa appears intact; the surface epithelium is continuous and fully restored; glandular funds appear tubular (d); various fibroblasts are evident (e); the gasric mucus occupies glandular pits (f); tonaca propria appears without dilatations; and interglandular spaces are regular (20×).

time but also recovery of all of the substances, even those present in the peel. For these reasons, we carried out an analysis, which revealed that *O. ficus indica* juice is a good source of vitamin C and contains a group of natural antioxidants, betalains included, which could act synergistically (*27*, *28*). The juice also contains ferulic acid and some flavonols, such as rutin and various isorhamnetin derivatives. These compounds are present mainly as water soluble glycosides; for this reason, the whole juice and the aqueous fraction have a significant antioxidant activity, whereas the organic fraction fails to show this activity. There is evidence that ascorbic acid plays a minor role in the total antioxidant efficiency of juices; in fact, it has been evaluated that the contribution of vitamin C to the total antioxidant activity of a fruit is usually less than 15% (*29*), but the presence of a good amount of polyphenolic compounds, especially flavonol glycosides, could contribute to the high level of antioxidant capacity also determined in this study.

This study clearly demonstrates an evident protective activity of *O. ficus indica* juice against ethanol-induced ulcer. The effect probably depends on the antioxidant activity of the phytochemicals of the juice, which are effective radical scavengers.

Ethanol produces gastric mucosal injury probably by accumulating toxic free radicals in the mucosal gastric cells. The initial event could be the disruption of the mucous and epithelial cells, with extensive sheets of necrotic cells detached from the viable pit cells. The concomitant destruction of vascular endothelium results in edema formation and stasis of gastric blood flow.

Pretreatment with *O. ficus indica* fruit juice preserves mucosal integrity, with continuous sheets of epithelium. Moreover, the mucus layer is restricted on the surface epithelial cells; therefore, the gastric mucosa remains epithelialized in spite of the presence of luminal ethanol. A normal feature of the mucosa is proven by straight glandular course with well-defined funds. Tonaca propria and muscolaris mucosae are normal. Ethanol-induced lesions are inhibited by agents that enhance mucosal defensive factors, such as sucralfate (*30*), but our results show that the mucus arrangement is different after pretreatment with *O. ficus indica* fruit juice from that with sucralfate. The observed activity could be dependent on the compounds with radical scavenging properties, which make them able to neutralize reactive species, harmful to the mucosa. The flavonoid fraction consists of rutin and isorhamnetin derivatives, and information about absorption and metabolism of these flavonols could explain better the mechanism of protective action. Absorption of glycoside derivatives in the small intestine is prevented because of their hydrophilic nature and their relatively high molecular weight. Moreover, the aglycones can be released by intestinal microflora that in addition, can cleave the pyrone ring producing phenyl acetic and phenyl propionic acid and other derivatives (*31*). Rutin is metabolized to quercetin, and isorhamnetin derivatives are metabolized to isorhamnetin. Isorhamnetin, which is the 3-methylated form of quercetin, and quercetin in itself, are not further metabolized (*32*). As aglycones, the flavonols can be absorbed; therefore, they can produce systemic effects. The capability of flavonoids to increase vascular tone and to regulate microcirculation could be involved in the protection of gastric mucosa (*33*, *34*).

Flavonoids stimulate the production of prostaglandins in isolated cells of gastric mucosa (*35*); therefore, the flavonols of *O. ficus indica* juice could exert antiulcer activity by means of the prostaglandins, which promote mucus and bicarbonate secretion, enhance mucosal blood flow, and reduce the increased microvascular permeability (*36*). Moreover, it is well-known that some flavonoids, like quercetin and rutin, are able to relax smooth muscle (*37*); therefore, ethanol-induced contraction of gastric smooth muscle (*38*) could be prevented by *O. ficus indica* fruit juice. The relaxation of the gastric muscles decreases gastric motility and reduces the volume of the necrotic agent on rugal crest (*38*). This action could play a role in the prevention of experimental ulcer (*39*).

Literature data report that flavonoids show cytoprotective activity by affecting various steps in the arachidonate cascade via the cyclooxygenase pathway (*40*) and probably exert a selective action on COX II without an important effect on COX I present in the gastric mucosa (*41*). Therefore, the flavonoids could inhibit the responce to phlogistic stimuli and our experimental observations also showed an antiinflammatory activity of the juice (unpublished results). In conclusion, *O. ficus indica* fruit juice is a rich source of micronutrients, including flavonoids, that play a role in health, including but not limited to their role as antioxidants. The dietary intake of *O. ficus indica* whole juice could be a good alternative to the daily intake of

five portions of vegetables, the amount recommended by the World Health Organization.

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