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Evaluation of antioxidant, antiinflammatory, and gastroprotective properties of *Rubus fruticosus* L. fruit juice

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The juice of *R. fruticosus* (RFJ) fruits grown in Sicily was analysed for polyphenol compounds and tested to evaluate *in vitro* antioxidant and *in vivo* antiinflammatory and gastroprotective effects. RFJ, containing mainly anthocyanins, such as cyanidin derivatives, significant amounts of phenolic acids, and smaller amounts of flavonoids, showed significant antioxidant activity in DPPH (2,2-diphenyl-1-picrylhydrazyl radical) (4,147.194 ± 17.199 mg trolox equivalent [TE]/100 ml), TE antioxidant capacity (8,312.444 ± 43.055 mg TE/100 ml), ferric reducing antioxidant power (2,177.830 ± 21.015 mg TE/100 ml), oxygen radical absorbance capacity (95,377.674 ± 616.194 μmol TE/100 ml juice), and β-carotene bleaching (72% ± 4.58) assay. *In vivo* studies showed that RFJ inhibit significantly the carrageenan-induced paw oedema (63–71%) in rats and possess antiinflammatory effects particularly significant in association with phenylbutazone (94–96%). In addition, RFJ pretreatment was able to prevent the ethanol-induced ulcerogenic effect in rats. All *in vivo* results were corroborated by histopathological observations and are in good agreement with antioxidant activity, confirming the relationships between biological effects observed and radical scavenging properties of RFJ.

KEYWORDS

anthocyanins, antiinflammatory activity, antioxidant activity, gastroprotective effect, polyphenols, *Rubus fruticosus* L.

1 | INTRODUCTION

Rubus fruticosus L. (Rosaceae) is an Armenian shrub, now widespread throughout Europe, Asia, Oceania, and North and South America (Zia-Ul-Haq, Riaz, De Feo, Jaafar, & Moga, 2014), well known for its delicious fruits, commonly known as blackberries. They are very delicious despite having low energy and are considered among the best dietary sources of various bioactive compounds such as phenolic acids, flavonoids, anthocyanins, carotenoids, tannins, vitamins, and minerals (De Souza et al., 2014; Namiesnik et al., 2014; Slatnar, Jakopic, Stampar, Veberic, & Jamnik, 2012). Worldwide commercial production of blackberry attested around 154,578 tons annually, and North America, Europe, and Asia are the main producers (Kaume, Howard, & Devareddy, 2012). These fruits are usually consumed fresh or processed as jam, tea, desserts, jellies, and bakery products. In addition, its pigments are used as natural food dyes in baked products, jellies, chewing gums, and beverages (Zia-Ul-Haq et al., 2014). The traditional use of *R. fruticosus* as food and medicinal plant is well known from ancient times, and the most important biological activities ascribed to it are antimicrobial, antioxidant, antiinflammatory, and anticancer (Kaume et al., 2012; Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015; Zia-Ul-Haq et al., 2014). Several studies showed that these health properties are due in particular to synergic effects of its various bioactive constituents and antioxidant components although polyphenols seem to play a pivotal role (Aragona et al., 2017; Kaume et al., 2012; Marino et al., 2013; Skrovankova et al., 2015; Smeriglio, Barreca, Bellocco, & Trombetta, 2016, 2017; Smeriglio, Monteleone, & Trombetta, 2014). Several factors such as cultivar, ripeness, agroclimatological conditions, and processing method affect the profile and intensity of these health properties (Kaume et al., 2012; Skrovankova et al., 2015; Zia-Ul-Haq et al., 2014).

Although many traditional uses of *R. fruticosus* have been verified *in vitro*, *in vivo* preclinical and clinical studies are still lacking and became necessary to assess its safety and efficacy.

In this manuscript, the polyphenolic profile as well as the *in vitro* antioxidant activity and the antiinflammatory and gastroprotective properties of a *R. fruticosus* juice (RFJ) in rats were investigated.

2 | MATERIALS AND METHODS

2.1 | Plant material and sample preparation

Cultivated *R. fruticosus* L. (Rosaceae) fruits were collected on Nebrodi Mountains (Messina, Sicily). A voucher specimen was deposited in the Herbarium of our Department (Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences, University of Messina).

Five hundred and fifty grammes of RFJ was obtained from 1 kg of fruits by a juice processor (yield 55%). It was centrifuged at 3,000 g for 15 min at 4 °C (Neya 10R, REMI), and the supernatant was aliquoted and stored at −80 °C until analysis to avoid nutritional and organoleptic depletion and to block the enzymatic activity. The juice did not further processed more than filtration on 0.22-μm nylon filter before injection into HPLC system.

2.2 | Animals

The biological activities of RFJ were tested on male Wistar rats (Charles River, Italia) weighing 180–200 g, kept in controlled conditions (temperature 22 ± 2 °C; relative humidity 60 ± 4%, in a 12-hr light/dark cycle), and fed on a standard diet (S. Morini Mill rat GLP, S. polo d'Enza, RE, Italia) and water ad libitum.

Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M: 116192), as well as with the The European Union regulations (EEC) (O.J. of E.C.L. 358/1 12/18/1986).

2.3 | Chemicals

All reagents and solvents used were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile, acetic, and phosphoric acids were HPLC-grade and were purchased from Merck (Darmstadt, Germany). The polyphenol reference compounds were purchased from Extrasynthese (Genay, France).

2.4 | Determination of proanthocyanidins content

The proanthocyanidins content was determined according to Tomaino et al. (2010). Briefly, an aliquot of RFJ (diluted 10 times with 0.05 M H_2SO_4 in order to obtain a final volume of 2.0 ml) was loaded onto a conditioned Sep-Pak C18 column, preconditioned with 2.0 ml of 5 mM H_2SO_4 and purged with air. The proanthocyanidins-rich fraction was eluted with 3.0 ml of MeOH and collected in a 100-ml flask containing 9.5 ml of absolute EtOH, shielded from light. To this mixture, 12.5 ml of $FeSO_4 \bullet 7H_2O$ solubilized in HCl 37% (300 mg/L) was added; the flask was placed in a boiling water bath and refluxed for 50 min,

after which it was rapidly cooled by immersion in cold water (20 °C). After 10 min, the absorbance at 550 nm was recorded on a UV–Vis spectrophotometer (Shimadzu UV-1601). The basal anthocyanins content of sample was obtained detracting the absorbance of sample prepared under the same conditions but cooled in ice and not warmed. Proanthocyanidins content was conventionally expressed as five times the amount of cyanidin formed, by means of a calibration curve with cyanidin chloride (e = 34,700). Results were expressed as mg of cyanidin chloride equivalents (CyE)/100 ml of RFJ.

2.5 | Vanillin index determination

The vanillin index was determined following the method described by Tomaino et al. (2010). Briefly, 2.0 ml of RFJ (diluted in order to avoid any interference), added with 0.5 M H_2SO_4 in order to obtain a final reading between 0.2 and 0.4 AU, was loaded onto a conditioned Sep-Pak C18 column. The column was washed with 2.0 ml of 5.0-mM H_2SO_4 and purged with air, and the sample was eluted with 5.0 ml of MeOH into a test tube. One millilitre of the methanol eluate was placed in a test tube (shielded from light), together with 6.0 ml of 4% vanillin methanol solution, and immersed in a water bath at 20 °C for 10 min. After cooling, 3.0 ml of HCl 37% was added. After precisely 15 min, the absorbance of sample was recorded on a UV–Vis spectrophotometer (Shimadzu UV-1601) at 500 nm against a blank. Catechin was used as reference compound (0–500 μg/ml), and results were expressed as mg of catechin equivalent (CatE)/100 ml of RFJ.

2.6 | Phenolic acids and flavonoids determination by RP-HPLC-DAD

The qualitative and quantitative determination of polyphenols was carried out according to Barreca et al. (2016), with some modifications, using an Agilent HPLC system (1100 series) equipped with a UV–Vis photodiode-array detector (DAD; G1315), a control system (G1323), an LC pump (G1312), and an autoinjector (G1313). The chromatographic separation was obtained using a Prodigy ODS3 column (250 mm × 4.6 mm, 5 μm; Phenomenex) with Solvent A (water/acetic acid, 97/3, *v/v*) and Solvent B (methanol) under the following conditions: 0–3 min, 0% B; 3–9 min, 3% B; 9–24 min, 12% B; 24–30 min, 20% B; 30–33 min, 20% B; 33–43 min, 30% B; 43–63 min, 50% B; 63–66 min, 50% B; 66–76 min, 60% B; 76–81 min, 60% B; 81–86 min, 0% B; and equilibrated 4 min for a total run time of 90 min. Flow rate was 1.0 ml/min, injection volume was 20 μl, and the column was thermostated at 25 °C. UV–Vis spectra of polyphenols were recorded from 190 to 400 nm. For quantitative analysis, the absorbance was acquired at 260 nm for phenolic acids and flavones, 292 nm for flavanones and 370 nm for flavonols. The peak's identity was confirmed by comparing their retention times and absorption spectra with those of pure (≥99%) commercially available standards. Quantification was carried out by external standard calibration curves. Results were confirmed by LC-DAD-MS analysis (Supporting Information).

2.7 | Anthocyanins determination by RP-HPLC-DAD

The qualitative and quantitative determination of anthocyanins present in RFJ was carried out according to Bellocco et al. (2016) using the instrument described in the previous section (Section 2.6) and a Chromolith Performance RP-18 column (100 mm × 4.6 mm; Merck) with Solvent A (4% phosphoric acid solution) and Solvent B (acetonitrile) as mobile phase. The elution gradient programme started with 95% A to reach 85% A and 15% B at 60 min and equilibrated 10 min for a total run time of 70 min. The flow rate was 0.75 ml/min, injection volume was 20 µl, and the column was thermostated at 35 °C. Detection was performed at 520 nm. UV–Vis spectra of anthocyanins were recorded from 200 to 600 nm. Peak identity was confirmed by comparing their retention times and absorption spectra with those of pure (≥99%) commercially available standards; quantification was carried out by external standard calibration curves.

2.8 | Determination of antioxidant and free radical scavenging properties

2.8.1 | Determination of total phenols content

The total phenols content in RFJ was determined according to Trombetta et al. (2010). Briefly, 50 µl of juice was added to Folin–Ciocalteu reagent (500 µl) followed by deionized water (450 µl). After 3 min, sodium carbonate (500 µl, 10% w/v) was added; samples were left in the dark at room temperature for 1-hr vortexing every 10 min, and the absorbance was recorded at 785 nm, using a UV–Vis spectrophotometer (Shimadzu UV-1601). Gallic acid was used as reference compound (0–600 µg/ml), and results were expressed as mg of gallic acid equivalents/100 ml of RFJ.

2.8.2 | DPPH radical scavenging activity

The DPPH free radical scavenging activity was evaluated according to Bellocco et al. (2016). Freshly prepared DPPH methanol solution (10^{-4} M) was mixed with 37.5 µl of sample water solution, and the mixture vortexed for 10 s at room temperature. The decrease in absorption at 517 nm against blank was measured after 20 min using a UV–Vis spectrophotometer (Shimadzu UV-1601). Trolox was used as reference compound (0–200 µg/ml), and results were expressed as mg of trolox equivalents (TE)/100 ml of RFJ.

2.8.3 | Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain (1996) with some modifications. The daily fresh working FRAP reagent solution was warmed at 37 °C. Twenty-five microlitres of sample water solution was added to 1.5 ml of FRAP reagent, and the absorbance was recorded at 593 nm, by a UV–Vis spectrophotometer (Shimadzu UV-1601), after incubation time of 4 min at 20 °C, using the FRAP reagent as blank. Trolox was used as reference compound (0–100 µg/ml), and results were expressed as mg TE/100 ml of RFJ.

2.8.4 | TE antioxidant capacity assay

The TE antioxidant capacity assay was carried out according to Morabito et al. (2010). Briefly, the reaction mixture (4.3-mM potassium persulfate and 1.7-mM ABTS solution 1:5, v/v) incubated for 12–16 hr in the dark at room temperature was diluted before use with phosphate buffer (pH 7.4) in order to obtain an absorbance, at 734 nm, of 0.7 ± 0.02. Fifty microlitres of juice was added to 1 ml of reaction mixture and incubated in the dark at room temperature for 6 min; the absorbance was then recorded at 734 nm using a UV–Vis spectrophotometer (Shimadzu UV-1601). Trolox was used as reference compound (0–250 µg/ml), and results were expressed as mg of TE/100 ml of RFJ.

2.8.5 | Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity was evaluated according to Barreca et al. (2016). Briefly, 20 µl of sample water solution diluted in 75 mM phosphate buffer solution (pH 7.4) was mixed with 120 µl of fresh daily 117-nM fluorescein solution. After a preincubation time of 15 min at 37 °C, 60 µl of freshly daily AAPH solution (40 mM) was added. The fluorescence was monitored every 30 s for 90 min (λ_{ex}: 485; λ_{em}: 520) using a Fluorescence Plate Reader (Fluostar Omega, BMG Labtech). A blank, using phosphate buffer instead of sample and trolox standard solutions (10–100 µM), was also included in each assay. The oxygen radical absorbance capacity value, using the area under the fluorescence decay curves, was estimated; trolox was used as reference compound (0–100 µM), and results were expressed as µmoles of TE/100 ml of RFJ.

2.8.6 | β-Carotene bleaching assay

The experiment was carried out according to Germanò et al. (2013). A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 2 mg of β-carotene was dissolved in 10 ml of chloroform (HPLC grade); 2 ml of the carotene–chloroform solution was pipetted into a boiling flask containing 25-µl linoleic acid and 200-µl Tween 40. The chloroform was removed using a rotary evaporator at 40 °C for 5 min, and 100 ml of distilled water was added to the residue, slowly with vigorous agitation, to form an emulsion. Then, 5 ml of the emulsion was added to a tube containing 200 µl of RFJ. The absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50 °C, and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm after sample preparation (t = 0 min) and at 20 min intervals until the end (t = 120 min) of the experiment. Control samples consisted of 5 ml of the emulsion and 200 µl of distilled water. Butylated hydroxytoluene (BHT) 1 mg/ml was used as positive control. The antioxidant activity was expressed as a percentage of inhibition with respect to the control according to the following equation:

$$AA\% = (1 - DRS/DRC) \times 100$$

where $AA\%$ is the antioxidant activity and DRS and DRC are the degradation rates of β-carotene in the reaction mixture with and without the sample, respectively, obtained as follows:

$$DR = \ln(a/b) \times 1/t$$

where a is the initial absorbance at 0 min, b is the absorbance at 120 min, and t = 120 min.

2.9 | Antiinflammatory activity

2.9.1 | Carrageenan-induced paw oedema in rats

Antiinflammatory activity of RFJ was evaluated according to the method of Galati et al. (2008). The animals were randomly divided into four groups of 10 animals each, night fasted, and treated by gavage in the morning.

- Group I (control) received only the vehicle, 3 ml of water, for 9 days;
- Group II (reference drug) received 3 ml of water, for 9 days. On the ninth day, the rats received phenylbutazone (50 mg/kg in water 0.5 ml/100 g b.w.) 1 hr after the last water administration;
- Group III (treatment) received 3 ml of RFJ, for 9 days;
- Group IV (combination treatment) received 3 ml of RFJ for 9 days. On the ninth day, the rats received phenylbutazone (50 mg/kg in water 0.5 ml/100 g b.w.) 1 hr after the last juice administration.

On the ninth day, 1 hr after the last administration, oedema was induced in the right paw of each rat, by a subplantar injection of 0.05 ml of a 1% carrageenan suspension (BDH, UK), in all groups of animals.

Paw volume was measured by a water plethysmometer (Ugo Basile, 7150, Italy) before the treatment (V_0) and 1, 2, 3, 4, and 5 hr after carrageenan injection (V_1). The increase in volume was recorded as the volume of oedema and was determined for each rat. The percentage inhibition of oedema, in treated animals versus control, was calculated as follows:

$$\frac{(V1-V0)\text{control}-(V1-V0)\text{treated}}{(V1-V0)\text{control}} \times 100$$

2.9.2 | Light microscopy

After the paw oedema measures with plethysmometer, the rats were sacrificed using ether anaesthesia. Samples of inflamed paws were collected, cut and fixed with 4% (p/v) paraformaldehyde (Immunofix®, BIO-OPTICA Milano) in phosphate buffer 0.2 M for 4 hr at 4 °C. The samples were washed with phosphate buffer solution 0.2 M for 1 hr, for three times, dehydrated in graded ethanol (30–100°), and finally embedded in Bioplast® (BIO-OPTICA Milano). The serial sections of the paw (5 μm thick), obtained by a rotary microtome (LEIKA 2065 Supercut), were stained with haematoxylin–eosin (H/E).

All samples were observed and photographed with an optical microscope Axioshop, Zeiss, equipped with Sony® DSC-85 camera.

2.10 | Gastroprotective activity

2.10.1 | Ethanol-induced ulcer in rats

Gastroprotective activity of RFJ was evaluated according to the method of Monforte et al. (2012) with some modifications. The animals were randomly divided into three groups of 10 animals each, night fasted and treated by gavage in the morning.

- Group I (control) received orally 3 ml of water, for 12 days. On the 12th day, 1 hr after the last water administration, the rats received by gavage the ulcerogenic agent EtOH 90% at a dose of 0.5 ml per rat;
- Group II (gastro-preventive treatment) received orally 3 ml of RFJ, for 12 days. On the 12th day, 1 hr after the last treatment with juice, the rats received by gavage the ulcerogenic agent EtOH 90% (0.5 ml per rat);
- Group III (reference drug) received orally 3 ml of water, for 12 days. On the 12th day, 1 hr after the water administration, the rats received by gavage sucralfate (Sucral, Bioprogress), as reference drug, at the dose of 100 mg/kg suspended in water (0.5 ml/100 g b.w). After 60 min from the treatment, the rats received by gavage the ulcerogenic agent, EtOH 90% (0.5 ml per rat).

One hour later, all the rats were sacrificed using ether anaesthesia, and the stomachs were removed, opened along the great curvature, and delicately washed with saline solution, so as not to remove the mucus layer from the mucosa surface.

2.10.2 | Macroscopic observation

For the macroscopic observations, the number, lengths, and severity of ulcers were noted and scored on an arbitrary 0–6 point scale (Magistretti, Conti, & Cristoni, 1988). The ulcer index (U.I.) of each stomach was the sum of its scores and was reported as arithmetic means ± SD.

2.10.3 | Light microscopy

After the macroscopic observations, the stomachs were extended on a cork surface to avoid deformities. Small pieces of every stomach were cut and fixed with neutralized 4% (p/v) paraformaldehyde (Immunofix®, BIO-OPTICA Milano) in phosphate buffer 0.06 M, for 4 hr at 4 °C. The samples were washed with a phosphate buffer solution 0.2 M, were dehydrated in graded ethanol (30–100°), and finally, embedded in Bioplast® (BIO-OPTICA Milano). The stomach serial sections (5 μm thick) obtained by a rotary microtome (LEIKA 2065 Supercut) were stained with H/E for general histology. Another set of sections was stained using the reaction to periodic acid-Shiff (PAS) for glycoprotein histochemistry. Periodic acid Shiff reacts with mucopolysaccharides and produce a characteristic carmine colour. All samples were observed and photographed with an optical microscope Axioshop, Zeiss, equipped with camera Sony® DSC-85.

2.11 | Statistical analysis

Results were expressed as mean ± SD of 10 independent experiments and analysed by one-way analysis of variance. The significance of the difference from control group for each treated group was assayed by using Student–Newman–Keuls test using a SigmaPlot 12.0 software. Statistical significance was considered at $p < .01$.

3 | RESULTS AND DISCUSSION

3.1 | Phytochemical screening and determination of polyphenol profile by RP-HPLC-DAD analysis

A preventive phytochemical screening of RFJ highlighted its total phenols (2,114.756 mg/100 ml of RFJ), flavan-3-ols (3.304 ± 0.042 mg CAE/100 ml of RFJ), and proanthocyanidins (1.730 mg CyE/100 ml of RFJ) content (Table 1). The vanillin index/proanthocyanidin content ratio gives a rough estimate of the degree of polymerization (polymerization index). The low value (1.910) of polymerization index calculated in our work indicates that RFJ has largest amounts of monomeric molecules (Smeriglio et al., 2018).

These preventive results were confirmed by HPLC-DAD analysis (Figure 1), which showed a very high content of polyphenols (total amount, 1,890.632 mg/100 ml of RFJ) and particularly of anthocyanin glycosides (1,410.178 mg/100 ml of RFJ) > phenolic acids (478.223 mg/100 ml of RFJ) > flavonoids (2.231 mg/100 ml of RFJ; Table 2). The cyanidin-3-O-glucoside was the most abundant compound (1,309.806 mg/100 ml of RFJ) followed by gallic acid (315.951 mg/100 ml of RFJ), vanillic acid (99.886 mg/100 ml of RFJ), and malvidin-3-O-galactoside (95.84 mg/100 ml of RFJ; Table 2). These results are in accordance with previous analysis on raspberry (Rubus idaeus L.) juice that showed a dominance of anthocyanins and negligible amounts of tannins and derivatives, mainly present in press cake (98.0%, mainly ellagitannin including lambertianin C and sanguiin H-6; Sójka, Macierzyński, Zaweracz, & Buczek, 2016). This explain the loss of these substances during the juice processing, which remain entrapped in seeds and skin residues. In particular, an average of 68.1% of ellagitannins and 87.7% of flavanols were retained in press cake-seedless fraction (Sójka et al., 2016); for this reason, for example, we did not find catechin and epicatechin in the juice. In addition, a significant negative correlation was found between the molecular mass of ellagitannins and their transfer to juice (Sójka et al., 2016). This observation corroborate our results about the mainly presence of monomeric molecules in RFJ.

The richness in polyphenols makes the RFJ a very interesting functional food particularly for its high content of cyanidin-3-O-glucoside

and allows us to hypothesize a potential use of this matrix for nutraceutical employment. Beyond their use as food dyes, in fact, anthocyanins have been investigated for their biological and pharmacological properties and literature data have described a wide range of interesting activities involving several pathways often related with their antioxidant properties (Smeriglio et al., 2014, 2016a, 2016b, 2017; Smeriglio et al., 2016). Reactive oxygen species are in fact involved in the development of many human diseases, and consequently, antioxidants play a crucial role due to their ability to overcome oxidative injuries, modulating biological pathways, and membrane functionality (Barreca et al., 2016).

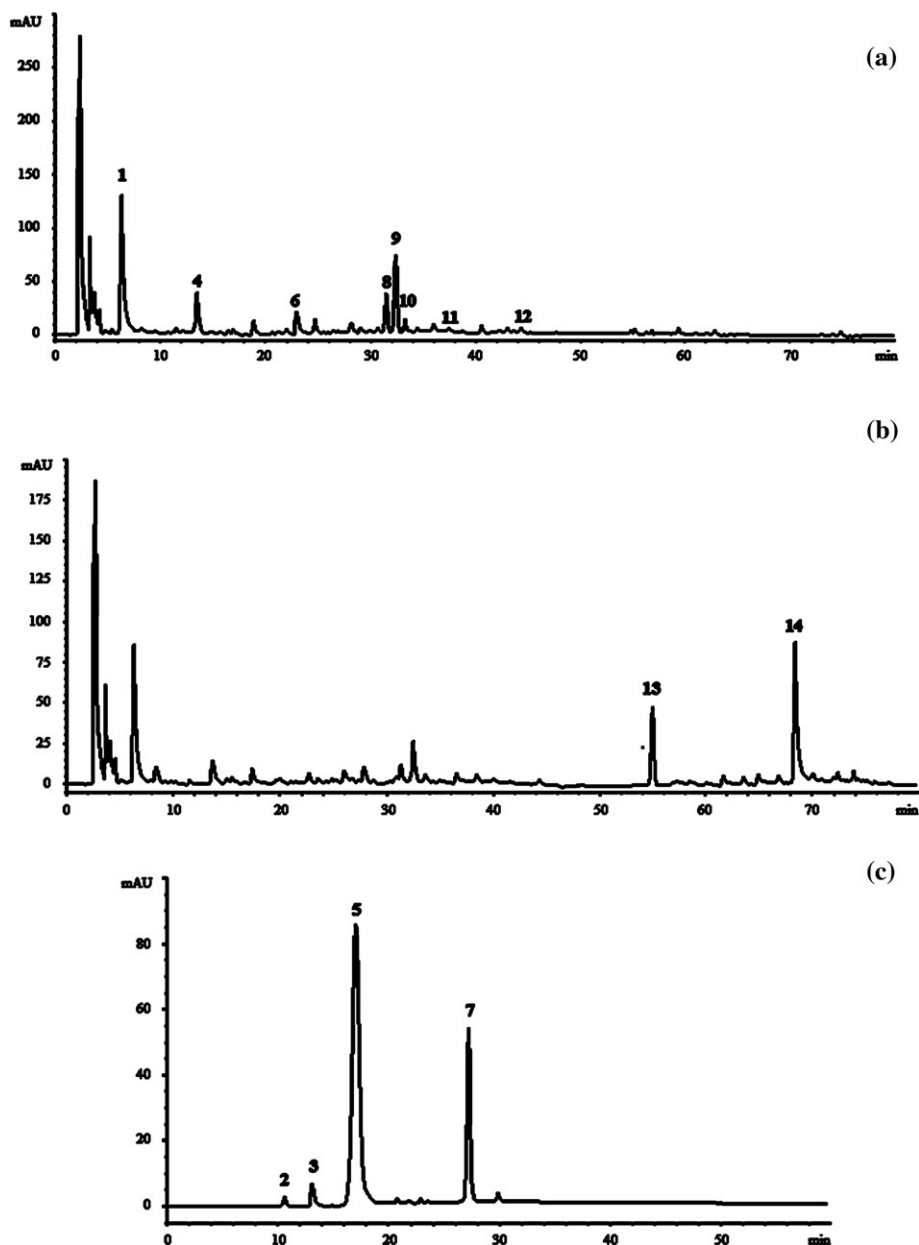## 3.2 | Antioxidant and free radical scavenging properties

In order to evaluate the antioxidant and free radical scavenging properties of RFJ and its behaviour under different reaction environments and mechanism typologies, several antioxidant assays (hydrogen atom transfer and electron transfer-based methods) were carried out.

The RFJ showed a remarkable dose-dependent antioxidant and free radical scavenging activity towards all assays performed (data not shown), with the following order of potency ORAC > TEAC > DPPH > FRAP as well as a strong capacity to inhibit linolenic acid oxidation (72% ± 4.58) with respect to the positive control (BHT, 82% ± 2.04; Table 1). Results suggest that RFJ acts as a powerful scavenger of several charged radicals, with a primary antioxidant activity that may be ascribed to the reducing and antiperoxidative ability, probably due mainly to hydroxyl groups linked to phenolic structures and their degree of glycosylation (Barreca et al., 2016; Bellocco et al., 2016; Smeriglio et al., 2016).

These abilities may have a potential beneficial role in the human defence system, where primary and relatively weak antioxidants can lead to the formation of more dangerous and reactive species such as hydrogen peroxide, peroxynitric radical, and hydroxyl radical (Bellocco et al., 2016).

Among the polyphenols identified, certainly the cyanidin-3-O-glucoside plays a crucial role in the antioxidant and free radical activity highlighted in the RFJ. Among anthocyanins of the same hydroxylation pattern in the A and C rings, in fact, compounds with only one OH group in the B ring (4′-OH; i.e., pelargonidin, malvidin, and peonidin) were found to possess lower antioxidant and free radical scavenging activity compared to cyanidin, characterized by a catechol structure. These characteristics confer to this molecule approximately the same antioxidant activity of quercetin, one of the most active flavonoids, demonstrating the importance of the unsaturation in the C ring, which allow electron delocalization across the molecule for radical stabilization (Bellocco et al., 2016).

About the antioxidant capacity of blackberry, a significant correlation was observed between total polyphenols and plasma total antioxidant capacity and/or total anthocyanins. However, the relationship between radical scavenger activity and total polyphenols seems to be closer than radical scavenging activity and total anthocyanins (Skrovankova et al., 2015). Therefore, both phenolics and anthocyanins influence antioxidant activity considerably. The greater antioxidant activity found in blackberry compared to other Rubus spp. is in fact

TABLE 1 Phytochemical screening and antioxidant properties of *Rubus fruticosus* juice (RFJ)

In vitro assay	RFJ
Total phenols (mg GAE/100 ml)	2,114.756 ± 27.296
Proanthocyanidins (mg CyE/100 ml)	1.730 ± 0.055
Vanillin index (mg CatE/100 ml)	3.304 ± 0.042
DPPH (mg TE/100 ml)	4,147.194 ± 17.199
FRAP (mg TE/100 ml)	2,177.830 ± 21.015
TEAC (mg TE/100 ml)	8,312.444 ± 43.055
ORAC (μmoles TE/100 ml)	95,377.674 ± 616.194
β-Carotene bleaching assay (AA%)	72 ± 4.58

Note. Data were expressed as mg/100 ml of RFJ and as means ± SD (n = 3) of three independent experiments. AA% = antioxidant activity; CatE = catechin equivalents; CyE = cyanidin chloride equivalents; FRAP = ferric reducing antioxidant power; GAE = gallic acid equivalents; ORAC = oxygen radical absorbance capacity; TE = trolox equivalents; TEAC = trolox equivalents antioxidant capacity.

FIGURE 1 Representative HPLC chromatogram of *Rubus fruticosus* juice acquired at 260 (phenolic acids), 292 (flavanones), and 520 nm (anthocyanins). Peak numbers referred to compounds showed in Table 1

correlated to the higher anthocyanin's and phenolic acids content, about 2.5 and 2 times greater respectively than raspberry (Skrovankova et al., 2015).

Other compounds, which certainly contribute to the antioxidant activity of RFJ, are phenolic acids. It is well known that the reducing ability of these compounds depends on the number of free hydroxyl groups in the molecular structure, which would be strengthened by steric hindrance (Smeriglio et al., 2018). According to this, among phenolic acids identified in RFJ, gallic acid plays a predominant role followed by protocatecuic and caffeic acids. All these compounds, as well as flavonoids (naringenin and naringenin-7-O-glucoside), contribute significantly to the antioxidant pattern of RFJ, although being a very complex matrix, the antioxidant properties of RFJ not always depend on the antioxidant activity of its main components and can be modulated by several mechanisms so that concepts of synergism and antagonism can be very relevant.

3.3 | Antiinflammatory activity

The carrageenan-induced paw edema in rats was the most used experimental model to investigate new antiinflammatory agents (Eddouks, Chattopadhyay, & Zeggwagh, 2012).

In our experimental model, we evaluated, for 5 hr, the antiinflammatory activity of RFJ alone and in combination with the standard reference drug phenylbutazone to find out any pharmacodynamics interactions. In the experimental conditions mentioned above (Section 2.9.1), no adverse effects were observed in all experimental groups.

To assess the antiinflammatory effects of RFJ, the paw tissues were examined by H/E staining. Control rats paw samples showed a widespread oedematous state in the epidermis and underlying dermis. The epidermis appeared flattened, and the typical feature of dermal papillae was altered. There was an increase of intracellular turgor between keratinocytes, and large spaces between the collagen fibre

TABLE 2 Polyphenol compounds identified and quantified in *Rubus fruticosus* juice (RFJ)

Compounds	Peak n.	R_t (min)	λ (nm)	mg/100 ml of RJ
Phenolic acids				
Gallic acid	**1**	6.666	270	315.951 ± 5.563
Protocatecuic acid	**4**	13.667	258; 294	30.732 ± 0.885
4-Hydroxybenzoic acid	**6**	23.228	255	10.132 ± 0.245
Chlorogeic acid	**8**	31.521	240;326	2.272 ± 0.075
Vanillic acid	**9**	32.214	260; 292	99.886 ± 0.655
Caffeic acid	**10**	33.26	234; 322	17.636 ± 0.484
Syringic acid	**11**	37.495	232; 274	1.236 ± 0.032
Cumaric acid	**12**	44.267	234; 310	0.378 ± 0.012
Flavanones				
Naringenin-7-*O*-glucoside	**13**	55,128	283; 332	0.327 ± 0.012
Naringenin	**14**	68,728	232; 290	1.904 ± 0.048
Anthocyanins				
Delphinidin-3-*O*-galactoside	**2**	10,594	276; 522	1.165 ± 0.044
Cyanidin-3-*O*-galactoside	**3**	13,499	280; 514	3.367 ± 0.056
Cianidin-3-*O*-glucoside	**5**	17,531	280; 514	1,309.806 ± 8.345
Malvidin-3-*O*-galactoside	**7**	27,555	284; 526	95.84 ± 1.223

Note. Data were expressed as mg/100 ml of RFJ and as means ± SD (*n* = 3) of three independent experiments. Bold values correspond to the peak numbers showed in Figure 1.

bundles in the dermis, which appeared disorganized, were observed. In addition, abundant presence of macrophages and dilated blood vessels were highlighted (Figure 2a).

Paw samples of rats treated with RFJ showed no histologic alteration and normal fibers showing a decrease of the oedematous state with a reduction of intercellular spaces and infiltrating inflammatory cells (Figure 2b). By comparing paw samples taken at the third and the fifth hour after carrageenan administration, it was evident that the antiinflammatory activity occurred as early as the third hour of treatment and became more evident at the fifth hour (Figures 2c,d).

Expressing the results as inflammatory activity inhibition % (Table 3), all treated groups showed a statistically significant inhibition of inflammatory activity with respect to control group (*p* < .001). Furthermore, a statistically significant inhibition of inflammatory activity by RFJ-treated group with respect to the phenylbutazone-treated group at the first hour was observed. However, what is even more interesting is that the combination treatment (RFJ-phenylbutazone) showed already at first hour the maximal antiinflammatory activity that was kept more or less constant for all 5 hr. In light of this, an additive-type pharmacodynamics interaction could be speculated (Table 3).

The antiinflammatory effects observed in our experiments could be due to the high amount of antioxidant compounds available in the RFJ. The strong antiinflammatory effect of cyanidin-3-*O*-glucoside, the major compound identified in RFJ (Table 2), has been widely reported and seems to involve a crosstalk between the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and the redox-dependent Nrf2 pathway (Ferrari et al., 2017). Furthermore, several studies showed the ability of cyanidin-3-*O*-glucoside and derivatives as well as purified anthocyanin fractions obtained from different anthocyanin-rich foods (cherries, bilberries, blueberries, cranberries, etc.) to decrease the cyclooxygenase COX-1 and COX-2 activity as well as protein expression levels of COX-2, NF-κβ, and various

interleukins (Smeriglio et al., 2014). Moreover, in several cell-based models, it seems that they could also inhibit human prostaglandin synthase activity (Smeriglio et al., 2014).

However, a synergistic interaction among the polyphenols identified in RFJ could enhance its bioactivity. In fact, the inhibition of proinflammatory responses (NO, iNOS, COX-2, and PGE$_2$) by polyphenols contained in Mexican blackberry (*Rubus* spp) extracts has been widely demonstrated, highlighting a significant correlation between antioxidant capacity and NO inhibition (Cuevas-Rodríguez et al., 2010). Thus, the antioxidant and antiinflammatory properties of polyphenols, in agreement with previous studies, could be attributed to the reduction of the nitrosative stress and subsequent formation of NO (Impellizzeri et al., 2015, 2015; Mandalari et al., 2011, 2011; Paterniti et al., 2017).

In addition, many phenolic compounds prevent neutrophil infiltration in the inflamed area and neutralize free radical species acting as antiinflammatory agents (Monforte et al., 2014).

3.4 | Gastroprotective activity

In the experimental conditions mentioned above (Section 2.10.1), no adverse effects were observed in all experimental groups.

The RFJ gastroprotective properties evaluation showed that control rats that received only ethanol showed intense and widespread gastric hyperaemia and thickened lesions with a U.I. of 8.56 ± 1.4.

The pretreatment with RFJ revealed a protective action against ethanol-induced ulcer. The stomach showed an aspect close to physiological state. In fact, a significant reduction in gastric hyperaemia in both number and severity of the lesions was observed. The U.I. significantly decrease to 1.46 ± 1.3 (*p* < .001) with respect to control (8.56 ± 1.4) nearly reaching the U.I. value of rats treated with sucralfate (1.25 ± 1.2).
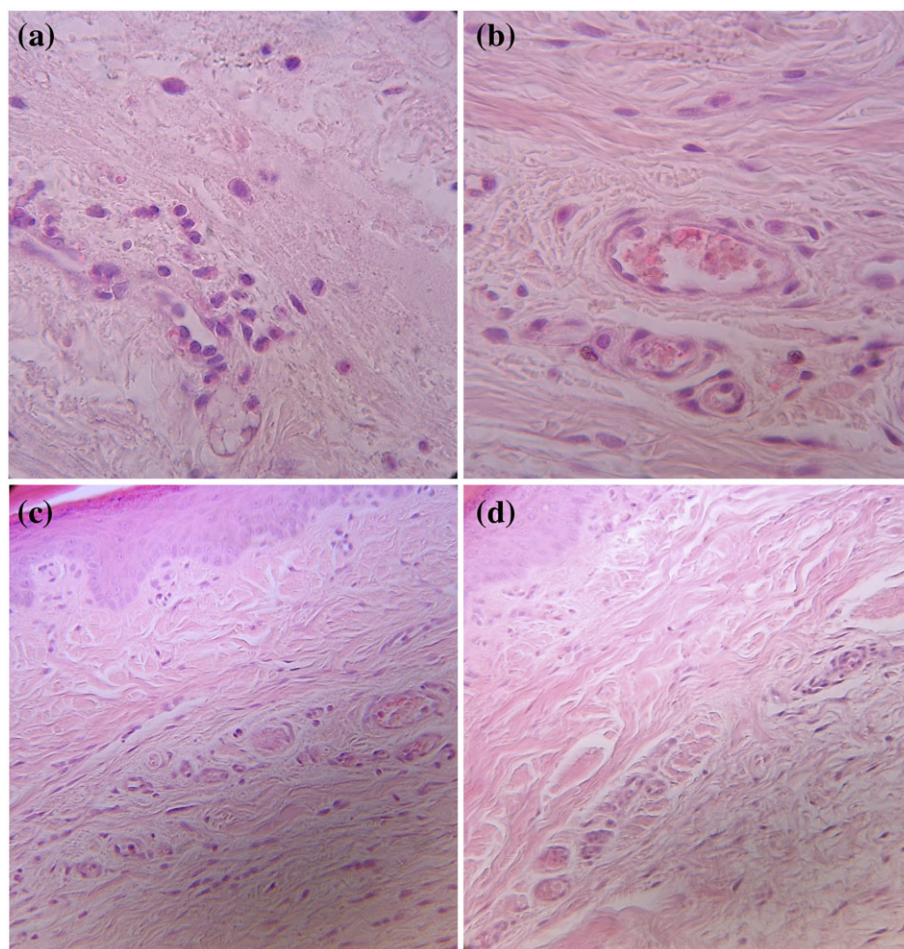
FIGURE 2 Light microscopy images of paw skin, stained with haematoxylin–eosin, of controls (a, magnification 100×) and rats treated with RFJ (b, magnification 100×), RFJ after 3 hr of carrageenan administration (c, magnification 40×) and RFJ after 5 hr of carrageenan administration (d, magnification 40×). Panel (a) shows an altered dermis with collagen fibre bundles disorganized, with presence of macrophages and dilated and tortuous blood vessels. Panel (b) shows a dermis well organized, with regular collagen fibers and blood vessels. Panel (c) shows a retrieval of skin morphology. Keratinocytes appear regular distributed in all layers, and dermo-epidermal junction is continuous. In the dermis, it is possible to observe some infiltrating cells and a good distribution of collagen fibers. Panel (d) shows a morphologic feature similar to which observed in (c) with less macrophages between collagen fibers [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Effect of RFJ on the carrageenan-induced paw oedema in rats

Treatment	Dose	% inhibition (±SD)				
		1 hr	2 hr	3 hr	4 hr	5 hr
RFJ	3 ml/die	64.31* (±1.85)	54.59* (±2.15)	55.24* (±2.24)	58.16* (±1.92)	70.78* (±2.08)
Phenylbutazone	50 mg/kg	28.47*,** (±1.05)	58.23* (±1.25)	62.19* (±1.12)	62.31* (±1.32)	63.07* (±1.16)
RFJ Phenylbutazone	3 ml/die50 mg/kg	96.19*,*** (±1.29)	93.19*,*** (±1.27)	93.41*,*** (±1.33)	93.26*,*** (±1.15)	94.06*,*** (±1.34)

Note. RFJ = *Rubus fruticosus* juice.

*$p < .001$ versus control group;

**$p < .001$ versus RFJ-treated group;

***$p < .001$ versus RFJ and phenylbutazone-treated groups.

Morphological study on gastric mucosa of control rats showed a typical feature of the ethanol-induced ulcer (Monforte et al., 2012).

Gastric mucosa samples, stained with H/E, showed tubular glands in the *tonaca propria* with dilated lumen, thickened wall, and cell population with signs of cytoplasmic suffering (Figure 3a). The apex of the glands was dilated, and apical desquamation was evident. A large number of widely dilated blood vessels around the gland fundi were observed (Figure 3a). Gastric mucosa samples of control rats stained with PAS showed the lack of mucous production, although a certain amount of neutral mucopolysaccharides in some neck cells was observed (Figure 3b).

Gastric mucosa samples of rats treated chronically with RFJ revealed a regular pattern of the *tonaca propria* glands that appeared straight with normal features and glandular lumens and fundi not dilated. There was a good appearance of parietal and principal cells. The glandular edges, the surface, and neck cells were regularly
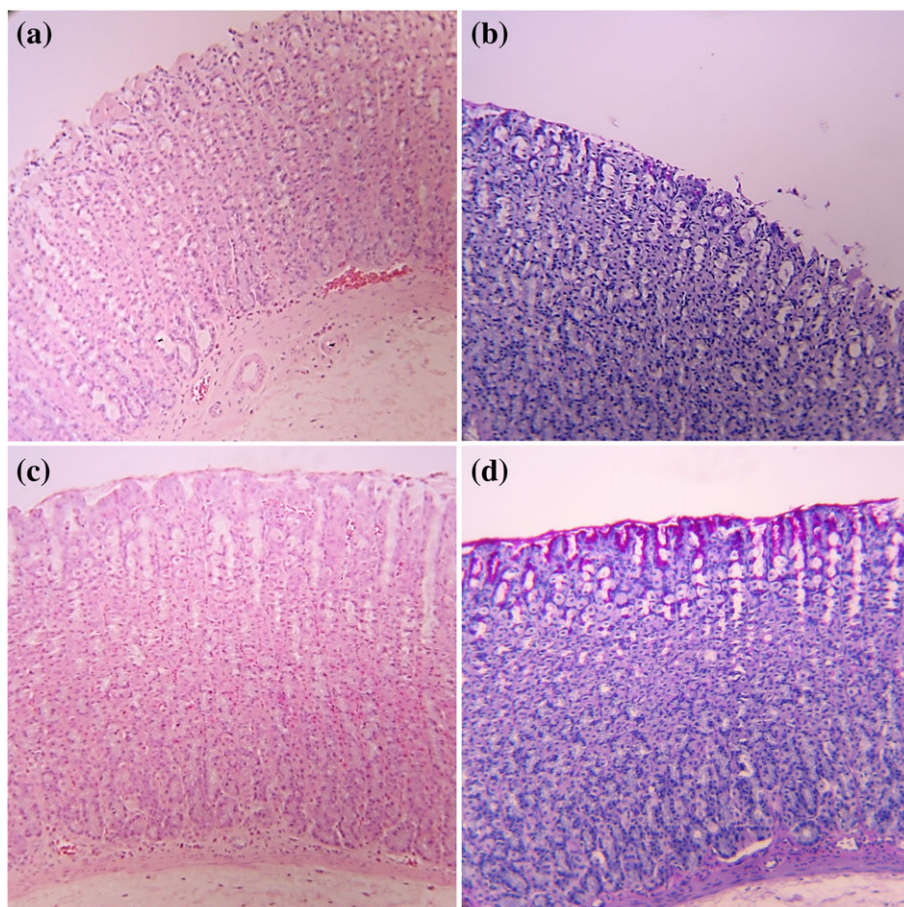
FIGURE 3 Light microscopy images (magnification 20×) of gastric mucosa of controls (a,b) and rats treated with RFJ (c,d) stained with haematoxylin–eosin and periodic acid-Shiff (PAS), respectively. Panel (a) shows a typical superficial erosion, glands with dilated lumen, and thickened wall. Dilated blood vessels around the gland fundi were observed. Panel (b) shows a decrease of mucus production. Panel (c) shows regular pattern of the glands that appear straight and glandular fundi not dilated. No large blood vessels around glandular fundi. Panel (d) shows a good appearance of parietal and principal cells. The glandular edges, the surface, and neck cells are regularly organized, with PAS-positive substance in the cytoplasm and covered with a thin layer of mucus [Colour figure can be viewed at wileyonlinelibrary.com]

organized with PAS-positive substance in the cytoplasm; moreover, a thin layer of mucus was found. Dilated blood vessels around glandular fundi in the *tonaca propria* were not observed (Figures 3c,d).

Dietary polyphenols with multiple biological mechanisms of action play a pivotal role in the management of gastric ulcer increasing the mucosal prostaglandins content, decreasing the histamine secretion from mast cells, and inhibiting the acid secretion (Farzaei, Abdollahi, & Rahimi, 2015). Other than a directly antioxidant activity, scavenging several charged oxygen, and nitrogen radicals, polyphenols exert their activity by several mechanisms.

Anthocyanins, as delphinidin and cyanidin, which possesses an ortho-dihydroxyphenyl structure on the B-ring, decrease the COX-2 expression, the inducible and short-lived isoform, which is over expressed in inflammatory cells in response to endotoxins, cytokines, and nitrogen, through inhibition of the transcription of m-RNA and synthesis of COX-2 (Smeriglio et al., 2014). On the contrary, these anthocyanins are less active on the COX-1, the constitutive isoform mainly responsible for the synthesis of cytoprotective prostaglandins in the gastrointestinal tract (Smeriglio et al., 2014). The molecular basis of these effects are the ability of these molecules to block MAPK pathways with the attendant activation of NF-κB, C/EBPδ, and AP-1 (Smeriglio et al., 2014). The antiulcerogenic activity of some isolated

flavonoids like naringenin as well as the gastroprotective effects of berry extracts were already observed (Awaad, Al-Jaber, Moses, El-Meligy, & Zain, 2013; Chao et al., 2010; Nesello et al., 2017; Park, Kim, & Choi, 2012). The possible mechanism that could justify the observed antiinflammatory and gastroprotective effects of RFJ is the ability of some phenolic acids and flavonoids to inhibit both COX-2 and iNOS involved in the production of proinflammatory mediators, such as nitric oxide and prostaglandins, at the inflammatory site (Wu et al., 2016; Farzaei et al., 2015).

4 | CONCLUSION

This work provided for the first time the phytochemical screening and the polyphenol profile of RFJ derived from Sicilian grown fruits, highlighting the very high content of polyphenol compounds with cyanidin-3-O-glucoside as the most abundant compound, the noticeable antioxidant and free radical scavenging activity as well as the remarkable antiinflammatory and gastroprotective properties of this product. RFJ showed a significant inhibition of oedema, from the first (64.31%) to the fifth (70.78%) hour. Furthermore, the combination treatment with phenylbutazone produced a maximal antiinflammatory

effect already at the first hour (96.19%). Moreover, in our experimental conditions, the chronic treatment with RFJ has clearly demonstrated a significant protective activity against ethanol-induced ulcer with an effect very close to which observed with sucralfate. These results allow us to speculate that RFJ could be very useful in the long-term antiinflammatory treatment in order to preserve the health human status and suggest that RFJ phytocomplex acts synergistically in producing antiinflammatory and gastroprotective effects playing a role in health, including but not limiting to their role as antioxidant.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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