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ORIGINAL CONTRIBUTION

A flavonoid-rich extract from bergamot juice prevents carcinogenesis in a genetic model of colorectal cancer, the Pirc rat (F344/NTac-Apc^{am1137})

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Abstract

Purpose To determine the potential of a flavonoid-rich extract from bergamot juice (BJe) to prevent colorectal carcinogenesis (CRC) in vivo.

Main methods Pirc rats (F344/NTac-Apc^{am1137}), mutated in *Apc*, the key gene in CRC, were treated with two different doses of BJe (35 mg/kg or 70 mg/kg body weight, respectively) mixed in the diet for 12 weeks. Then, the entire intestine was surgically removed and dissected for histological, immunohistochemical and molecular analyses.

Results Rats treated with BJe showed a significant dose-related reduction in the colon preneoplastic lesions mucin-depleted foci (MDF). Colon and small intestinal tumours were also significantly reduced in rats supplemented with 70 mg/kg of BJe. To elucidate the involved mechanisms, markers of inflammation and apoptosis were determined. Compared to controls, colon tumours from BJe 70 mg/kg-supplemented rats showed a significant down-regulation of inflammation-related genes (*COX-2, iNOS, IL-1β, IL-6* and *IL-10* and *Arginase 1*). Moreover, in colon tumours from rats fed with 70 mg/kg BJe, apoptosis was significantly higher than in controls. Up-regulation of *p53* and down-regulation of *survivin* and *p21* genes was also observed.

Conclusions These data indicate a strong chemopreventive activity of BJe that, at least in part, is due to its pro-apoptotic and anti-inflammatory actions. This effect could be exploited as a strategy to prevent CRC in high-risk patients.

Keywords Bergamot juice · *Citrus bergamia* · Cancer · Colon carcinogenesis · Inflammation

Abbreviations

BJ	Bergamot juice
BJe	Flavonoid-rich extract from bergamot juice
SCID	Severe combined immunodeficiency
CRC	Colorectal cancer
NM	Normal mucosa
PCNA	Proliferating cell nuclear antigen
MDF	Mucin-depleted foci
FAP	Familial adenomatous polyposis
BW	Body weight

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Introduction

Citrus aurantium ssp. *Bergamia* Risso et Poiteau (*Citrus bergamia* or Bergamot; Rutaceae family) is a typical tree of the Calabria region, in southern Italy. Its fruits are mainly used for the extraction of the essential oil, widely employed in aromatherapy [1] and preparation of perfumes, cosmetics and food, as well as studied for its potential use in the health field [2, 3]. Instead, bergamot juice (BJ), obtained from squeezing the fruit endocarp, has been considered so far a by-product with waste disposal problems. Recently, we chemically characterized the flavonoid-rich extract from BJ (BJe) and documented its antioxidant and anti-inflammatory activities, both in vitro and in vivo [4]. Moreover, BJ and BJe have also shown to reduce the growth rate of various cancer cell lines through different molecular mechanisms depending on cancer type [5–7]. Furthermore, our recent results indicate that BJ decreased spontaneous neuroblastoma metastasis formation in the lung of severe combined immunodeficient (SCID) mice [8], instead, to date, no

studies have investigated the effect of BJe in in vivo models of cancer.

Colorectal cancer (CRC) is the third most frequent type of cancer in Europe, after prostate and breast cancers [9]. Despite improvements, chemotherapy is not fully successful, pointing the need of new strategies of prevention to reduce both the incidence and mortality. During past decades, polyphenolic-rich extracts from natural sources, have attracted attention as potential chemopreventive agents against CRC, since they are able to reduce colon carcinogenesis in vitro and in vivo [10].

Citrus fruits represent major eaten fruits worldwide and one of the most important dietary sources of flavonoids, along with tea, berries, red wine, apples, and legumes. Several experimental and observational studies strongly indicate that *Citrus* juices and their extracts can exert anti-cancer effects [11], mainly due to the ability of their flavonoids to interfere with intracellular signalling pathways linked to cancer initiation, promotion and progression [12]. Interestingly, a very recent meta-analysis has highlighted an inverse association between *Citrus* fruits and oral cancer risk [13].

Given the richness of BJe in polyphenolic compounds and its activity in cancer cell lines [5–7], we thought interesting to test BJe in the Pirc rat (F344/NTac-Apc^{am1137}), an animal model of colon carcinogenesis [14]. This rat strain carries a germline mutation in *Apc* gene, the key genetic event in both familial adenomatous polyposis (FAP) and sporadic CRC [14]. Remarkably, at variance with genetic models like *Apc-Min* mice developing tumours mostly in the small intestine, Pirc rats grow preneoplastic lesions such as mucin-depleted foci (MDF) and tumours also in the colon, thus resembling CRC and FAP. Potentially, it stands as a robust model to test compounds with chemopreventive activity on colon cancer [15, 16].

Based on these considerations, the aim of our study was to determine whether BJe administered to Pirc rats (F344/NTac-Apc^{am1137}) may affect colon tumorigenesis, also exploring the mechanism of action.

Materials and methods

BJe

The company "Agrumaria Corleone" (Palermo, Italy) provided the BJe, prepared with bergamot fruits harvested in the Reggio Calabria province (Italy). Liquid BJe was obtained by passing bergamot juice through columns equipped with adsorbent resins, known to retain polyphenols. These were then eluted with NaOH and immediately passed through cationic resins, thus obtaining the flavonoids in their acid form. The extract was then collected and lyophilized to obtain a dry powder that was stored at − 20 °C until further use. The BJe used in the present study was the same already employed in other experimental research. [7, 17–21]. Nevertheless, before beginning this study, we performed the quali-quantitative analysis of flavonoids present in BJe by HPLC, confirming the same chemical composition previously reported [7, 17]. The principal flavonoids of BJe are (mg/g) neohesperidin (98.31), naringin (92.68), melitidin (68.42), hesperetin (56.74), neoeriocitrin (48.16) and naringenin (36.14).

Animals and treatments

Pirc (F344/NTac-Apc^{am1137}) and wild type (WT) Fisher F344/NTac rats, originally obtained from Taconic (Taconic Farms, Hudson, NY, USA), were maintained and bred as reported [15] by mating heterozygous Pirc rats with WT rats; pups were genotyped at 1 month of age. Male Pirc rats, aged 4 weeks, were randomly assigned to the following groups: i) controls ($n = 10$), fed with a standard AIN-76 diet, ii) BJe 35 mg/kg of body weight (BW; $n = 10$) and iii) BJe 70 mg/kg BW ($n = 7$) that were fed with the same AIN-76 diet as controls, but supplemented with BJe, to provide a dose of 35 mg/kg or 70 mg/kg of BJe, respectively. These doses were chosen on the basis of previous carcinogenesis experiments with polyphenolic extracts from various sources, in which a dose of 50 mg/kg was used [22]. The diets containing BJe were prepared adjusting the quantity of BJe based on the BW determined each week. The number of rats to be allocated to the different groups was based on the variability of colon tumorigenesis in Pirc rats aged 16 weeks, on the expected chemopreventive effect of BJe and also, on the availability of BJe to give to the animals. Rats were euthanized by CO_2 asphyxia after 12 weeks of treatment (16 weeks of age), in line with the experimental protocol approved by the Commission for Animal Experimentation of the Italian Ministry of Health (Authorization number 323/2016-PR). Experiments were carried out in accordance with both the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and ARRIVE guidelines.

Processing of colon, sample collection and determination of tumours and mucin-depleted foci (MDF)

After euthanasia, the entire intestine was dissected, flushed with cold saline and longitudinally opened. Pirc rats at 16 weeks of age present some macroscopic tumours in the intestine which were enumerated at the opening of the intestine. After this evaluation, small samples (about 25 mg) were immediately taken from colon tumours as well from the apparently normal mucosa (NM) (scraped from the proximal part of the colon), put in RNAlater™ (RNA stabilization Reagent, Qiagen), and stored at − 80 °C until used

to determine gene expression [15]. A small sample (about 9 mm^2) of both NM and tumours from the medial portion of the colon was also collected, fixed in 10% formalin solution and processed for histology to assess proliferation, apoptosis and expression of CD68 and COX-2 in longitudinal sections (4 µm thick; see below). The remaining colon and rectum were fixed flat in formalin and stained with high-iron diamine Alcian blue (HID-AB) to determine the number of MDF (number of MDF/colon), as biomarkers of colon carcinogenesis [23]. Briefly, HID-AB technique stains mucin production, and it allows the visualization of MDF as focal lesions with absent or very small production of mucins in the whole unbedded colon, when observed at the microscope (40× magnification) [15, 23]. The determination of MDF in each sample was carried on by two observers on coded samples, as reported [15, 23].

Cell proliferation, apoptosis, expression of CD-68 and COX-2 in NM and colon tumours

Proliferative activity was assessed in the morphologically NM and colonic tumours, determining proliferating cell nuclear antigen (PCNA) immunoreactivity with a mouse monoclonal antibody (PC-10, Santa Cruz, CA, USA) at 1:1000 dilution, as previously reported [15]. Proliferative activity was expressed as labelling index (LI): number of cells positive to PCNA/cells scored x 100, evaluated in at least 12 full longitudinally sectioned crypts of the NM, and in at least 600 cells in case of tumours. CD68 and COX-2 expression was determined quantifying reactivity as the number of labelled cells/area scored evaluated with the ACT-2U software (Nikon, Instruments Europe, Badhoevedorp, NL) connected via a camera to the microscope (Optiphot-2, Nikon, NL), as reported [24]. These determinations were carried out blindly and independently by two observers.

Apoptosis evaluation was carried out in paraffin-embedded sections of colon tumours stained with hematoxylin–eosin and observed at the microscope to enumerate cells with the following characteristics of apoptosis: cell shrinkage, loss of normal contact with the adjacent cells of the crypt, chromatin condensation or formation of round or oval nuclear fragments ("apoptotic bodies"). At least 600 cells/sample were scored, and apoptosis was quantified using the apoptotic index (AI = number of apoptotic cells/cells scored × 100) by two observers blindly and independently [25].

Real-time PCR analyses

Expression of mRNA was assessed by real-time PCR. Total RNA from tissue samples was extracted using TRIzol reagent according to the manufacturer's protocol. Then, equal amounts of total RNA (2 µg) were reverse transcribed using high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative PCR reactions were set up in triplicate in a 96-well plate and were carried out in 20 µL reactions containing 1xSYBR® Select Master Mix (Applied Biosystems), 0.2 µM of primers and 25 ng RNA converted into cDNA. The primer sequences used for the real-time PCR analyses are listed in Table 1. Beta-actin was used as endogenous control. Data were collected and analysed using the $2^{-\Delta\Delta CT}$ relative quantification method.

Statistics

Data are presented as means ± SE. Differences in MDFs and tumours among the three different groups were analysed with one-way ANOVA followed by Tukey's multiple range test. Post-test for linear trend across different doses of BJe was carried out using GraphPad Prism 5.0 (GraphPad Software), as appropriate. When only two groups were considered (i.e., controls *vs* 70 mg/kg BJe), differences were

Table 1 Oligonucleotide primers used for the real-time PCR analyses

Gene products	Forward primer sequences	Reverse primer sequences
Survivin	CCGAGAATGAGCCTGATT	ACCTGCTTCTTGACTGTAA
p21	CACACAGGAGCAAAGTAT	CAAAGTTCCACCGTTCTC
p53	TACAAGAAGTCACAACACAT	AGATACTCAGCATACGGATT
BAX	CGATGAACTGGACAACAAC	CACGGAAGAAGACCTCTC
Bcl-2	GAGTGTCTCAGGCGAATT	TGTATCCACATCAGCAATC
COX-2	AGACACTCAGGTAGACAT	TGGTAGCATACATCATCAG
iNOS	TACTGCTGGTGGTTACAAG	GGTATGCCCGAGTTCTTT
IL-6	GACCATCCAACTCATCTT	CTTAGGCATAGCACACTA
IL-1β	AGCCAACAAGTGGTATTCT	ACAGGACAGGTATAGATTCTTC
IL-10	TGCGACGCTGTCATCGATTT	TTCATGGCCTTGTAGACACCTTT
Arginase 1	CAGTATTCACCCCGGCTACG	AGTCCTGAAAGTAGCCCTGTCT
β-Actin	TATGGAATCCTGTGGCATC	GTGTTGGCATAGAGGTCTT

analysed with *t* test. *P* values of ≤0.05 were considered statistically significant.

Results

Effect of BJe on the tumorigenesis of Pirc rats

At the beginning of the study, the average weight of the rats was 62 ± 2 g (mean ± SE, $n = 27$). Twelve weeks later, the average weight was similar among dietary groups (mean values ± SE were: 313 ± 11 g, 311 ± 6 g and 296 ± 5 g, in controls, BJe 35 and BJe 70 mg/kg groups, respectively), with no apparent sign of toxicity of the treatment.

Animals were euthanized when they were 16 weeks old. At this age, besides the microscopic lesions MDF, some macroscopic tumours were already present in both the colon and the small intestine. The number of these macroscopic lesions (total tumours) in the rats fed with 70 mg/kg BJe was significantly lower ($P < 0.05$) compared to controls (Fig. 1a), with a linear trend in the reduction of tumours across the different BJe doses ($P < 0.01$). Considering separately tumours in the colon (Fig. 1, panel b) or in the small intestine (Fig. 1, panel c), we also noticed that the group supplemented with 70 mg/kg of BJe had a significantly lower number of tumours than controls, whereas, differences between controls and the 35 mg/kg BJe group, although noticeable, did not reach the statistical significance. A linear trend across BJe doses was always present both in the colon and in the small intestine ($P < 0.05$).

After the enumeration of macroscopic tumours, colons were stained with HID-AB to determine the number of MDF in each group. The results of this analysis (Fig. 1d) showed that in animals treated with BJe (both doses), there was a statistically lower number ($P < 0.0001$) of these preneoplastic lesions when compared to controls. A significant linear trend ($P < 0.0001$) across the two doses of BJe was also observed (Fig. 1d).

Effect of BJe on inflammatory markers in Pirc rats

To understand the molecular mechanisms underlying the protective effect of BJe on tumorigenesis, we determined the expression of genes encoding *COX-2, iNOS, Arginase 1* as well as of some cytokines (*IL-1β, IL-4 IL-6* and *IL-10*) secreted by tumour and/or stromal cells in both normal mucosa and colon tumours of rats supplemented with 70 mg/kg of BJe or controls [26]. The results showed a significant down-regulation of these genes, ranging from 0.5- to 0.3-fold, in the tumours of BJe-treated animals compared to those from controls (Fig. 2). On the contrary, their

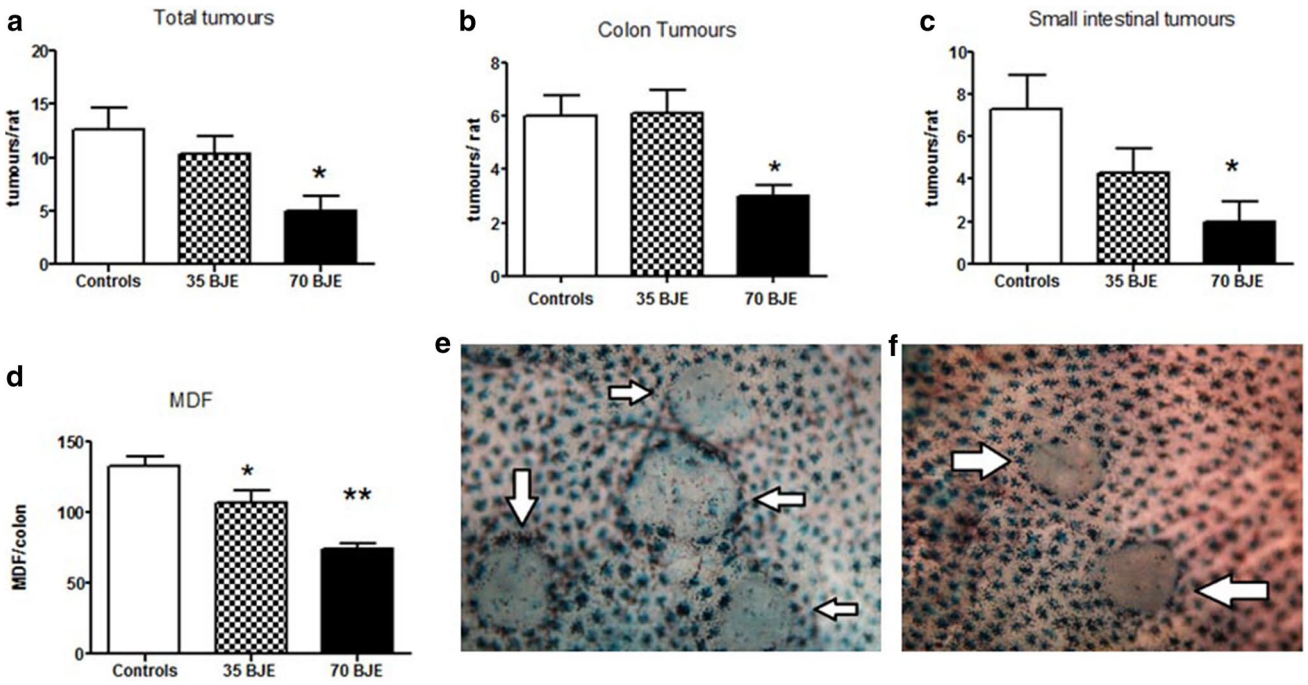

Fig. 1 Intestinal tumorigenesis in Pirc rats fed for 12 weeks with a control diet or with a diet supplemented with BJe at 35 mg/kg or 70 mg/kg BW. **a** Total intestinal tumours; **b**, colon tumours; panel, **c**, small intestinal tumours; **d**, MDF in the colon. Data are mean values ± SE. *, **$P < 0.05$ and $P < 0.01$ *vs* controls, respectively. **e** and **f**, topographical view of Pirc colons (from controls and BJe 70 mg/kg group, respectively) stained with HID-AB showing MDF (white arrows) (original magnification: ×40)

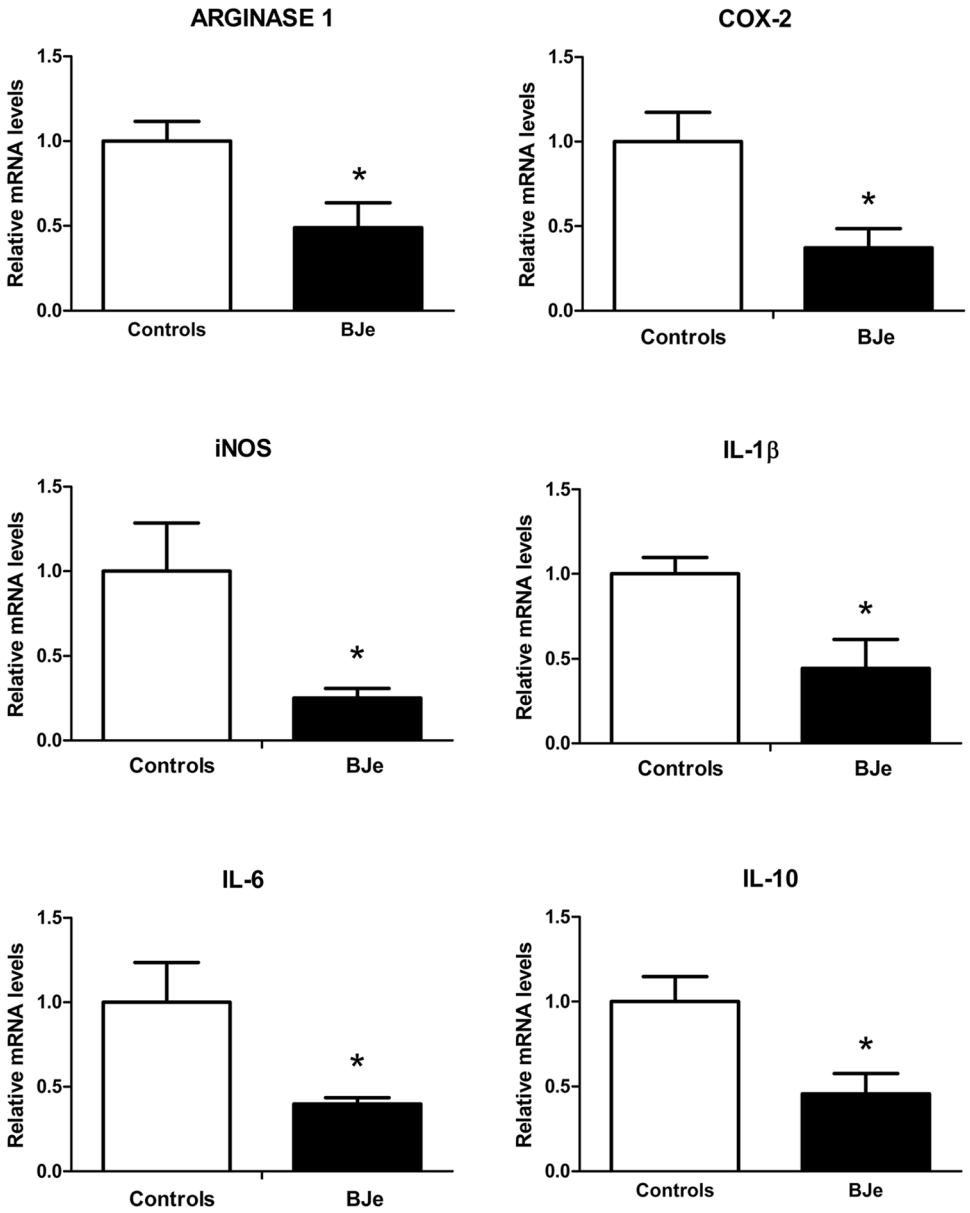

Fig. 2 mRNA levels of inflammation-related genes in colon tumours from controls (white bars) or 70 mg/kg BJe (black bars) group. Results of real-time PCR are expressed as relative fold change detected in animals supplemented with BJe compared to controls, after normalization against β-actin used as endogenous control. Values are mean ± SE ($n = 7$ and 6, for controls and BJe 70 mg/kg group, respectively). *$P < 0.05$ *vs* controls

expression in the NM was similar between the two groups (data not shown).

Immunohistochemical analyses to determine the expression of CD68-positive cells (macrophages) in the NM showed a similar expression of these cells in both groups, controls and 70 mg/kg BJe (Fig. 3, left panel). Conversely, the expression of COX-2 in NM cells of BJe-supplemented rats was lower, although not significantly, compared to controls (Fig. 3, right panel).

Effect of BJe on apoptotic markers in Pirc rats

To better clarify the anti-cancer effect of BJe, we also measured the expression of some genes related to apoptosis and involved in colon carcinogenesis. Significant variation in the expression of these genes was found in colon tumours (Fig. 4). In particular, compared to controls ($n = 7$), *p53* expression was significantly up-regulated in animals supplemented with BJe 70 mg/kg (1.6-fold, $n = 6$). Moreover, compared to controls, in BJe-treated groups, *BAX* and *Bcl-2* mRNA levels were increased (1.2-fold) and reduced (0.85-fold), respectively, although these differences did not reach statistical significance (Fig. 4). Furthermore, with respect to controls, the mRNA level of the anti-apoptotic gene *Survivin* was significantly reduced in animals supplemented with BJe 70 mg/kg (0.5-fold), as well as *p21* mRNA (0.35-fold). On the contrary, in the NM, the expression of the same genes was similar in both controls and BJe 70 mg/kg group (data not shown).

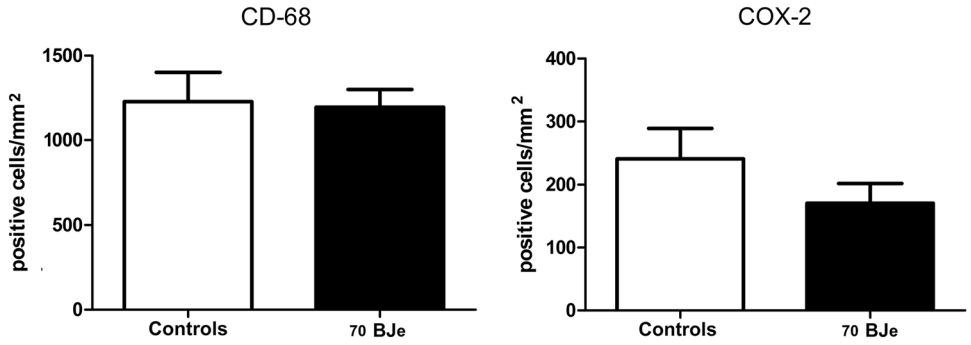

Fig. 3 Immunohistochemistry evaluation of CD68 (left panel) and COX-2 (right panel) expression in the NM of controls and rats supplemented with BJe. Data are mean values ± SE of cells labelled with specific antibodies for CD68 ($n = 6$ and 7 in controls and BJe group, respectively) and COX-2 expression ($n = 9$ and 6 in controls and BJe group, respectively)

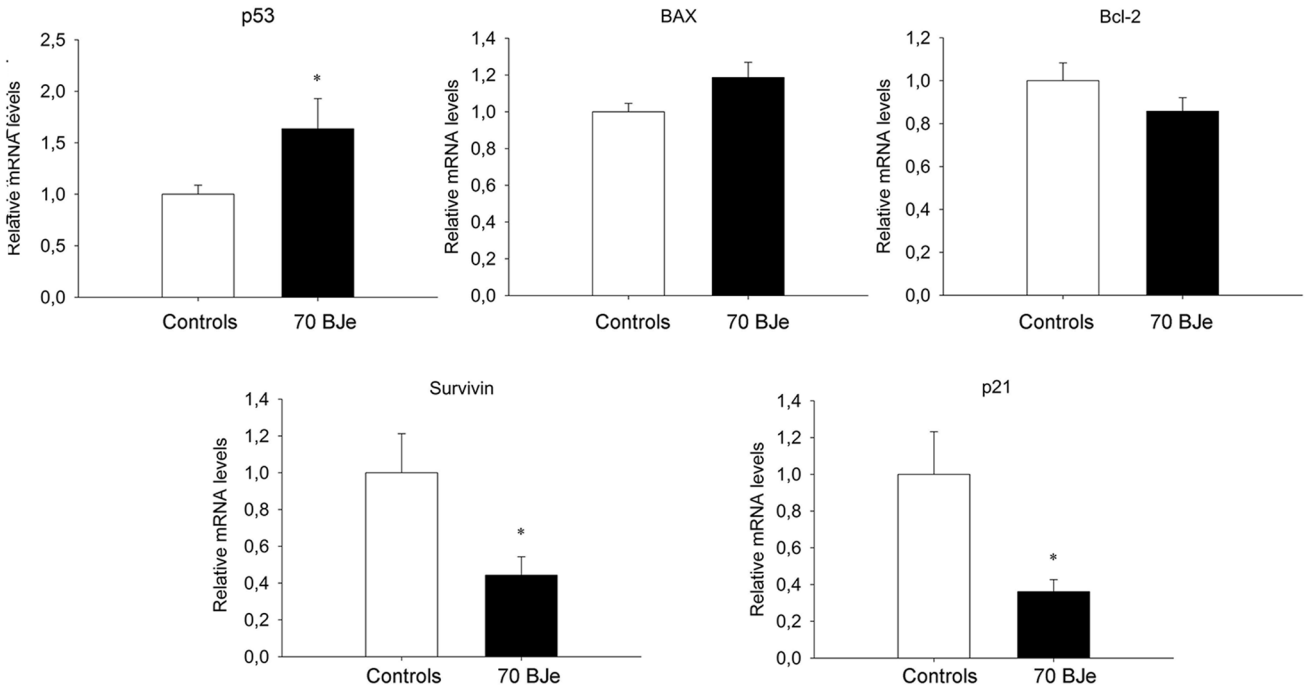

Fig. 4 Gene expression profiling of apoptosis-related genes in colon tumours from rats fed with a diet containing (black bars) or not (white bars) 70 mg/kg BW of BJe. Results from real-time PCR are expressed as relative fold change detected in animals from the BJe group compared to the controls, after normalization against β-actin used as endogenous control. Values are mean ± SE ($n = 7$ and 6 in controls and BJe 70 mg/kg group, respectively). *$P < 0.05$ *vs* controls

We also determined apoptosis in histological sections of the tumours: the results documented a significantly higher number of apoptotic cells in the tumours from BJe-treated rats (70 mg/kg) than in those from controls (Fig. 5).

On the contrary, cell proliferation, as determined evaluating PCNA immunostaining and expressed as LI, did not vary in the two groups. In the tumours, LI was 46.3 ± 3.9 ($n = 8$) and 44.3 ± 4.6 ($n = 6$) in controls and BJe-treated group, respectively. Similarly, in the NM, there was no difference in the proliferative activity between controls ($n = 7$) and BJe-treated ($n = 5$) rats (LI was 32.8 ± 3.9 and 31.5 ± 4.3 in controls and BJe-treated rats, respectively).

Discussion

The main finding of the present study is that BJe administered at two different dosages to Pirc rats reduces spontaneous tumorigenesis in this genetic model of CRC. Accordingly, a dose-related reduction in the number of the precancerous lesions MDF was found in the rats supplemented with BJe, together with a significantly lower number of intestinal tumours in the rats fed with the higher dose of BJe. To the best of our knowledge, this is the first

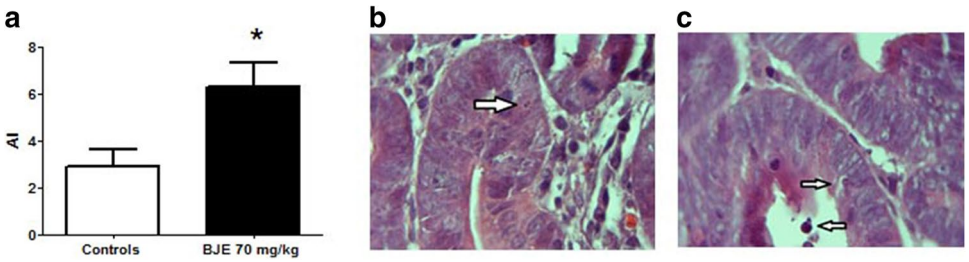

Fig. 5 **a**: apoptotic index (AI) in colon tumours from control rats or from rats daily fed with a diet contained 70 mg/kg BJe. Data are mean values ± SE ($n = 7$ and 6 for tumours in controls and BJe group, respectively). *$P < 0.05$ vs controls. **b** and **c**: histological sections of two colon tumours (from controls and 70 mg/kg BJe group, respectively) showing apoptotic cells (white arrows). Sections were stained with haematoxylin and eosin. Original magnification: × 1000

study demonstrating that an extract of BJ rich in flavonoids can prevent colon carcinogenesis in animals.

The Pirc rat carries a truncating mutation in *Apc* gene, considered crucial in sporadic CRC and causing hereditary colon cancer syndromes such as FAP [14]. Due to this genetic mutation, the Pirc rat develops spontaneous intestinal tumorigenesis, hence, it is considered an appropriate model to study and screen chemopreventive agents [16]. Numerous microscopic preneoplastic lesions (i.e., MDF) are present in the colon of this rat; these dysplastic lesions, characterized by a scant mucous production, are also formed treating rodents with specific colon carcinogens, and they have been described in humans at high risk of CRC, such as FAP patients [27, 28]. In addition, MDF are correlated with colon carcinogenesis, since their number is reduced in rats treated with chemopreventive agents and, conversely, it is increased with cancer-promoting diets [23, 29]. Therefore, their determination can be used as a biomarker in short-term carcinogenesis studies [23].

Mediterranean diet is known for its beneficial effects, and *Citrus* fruits represent an important constituent of it. Experimental and epidemiological studies indicate that *Citrus* juices and their extracts can contribute in maintaining a good health status, thanks to the capability of their bioactive molecules to modulate key intracellular pathways that play a pivotal role in degenerative processes [12, 30–34]. Our previous studies carried out using BJe with the same flavonoid composition of that employed in the present study, demonstrated its antimicrobial [35], antioxidant [17, 18] and anti-inflammatory effects both in cell cultures [19] and in animal models [20, 21, 36]. Regarding the anti-cancer activity, we recently showed that BJ exerts antiproliferative and pro-apoptotic effect in human hepatocellular carcinoma HepG2 cells [6], while in neuroblastoma cell lines, inhibits both growth and adhesiveness [5, 8], that can be responsible for the slight reduction of lung metastasis in a metastatic xenograft model [8]. Moreover, we showed that BJe inhibits the HT-29 human colorectal carcinoma cell proliferation through apoptosis triggered by different mechanisms depending on the extract concentration [7].

Prevention of CRC, using natural or synthetic agents, could be a promising strategy to slow or inhibit CRC [16]. Actually, since colorectal carcinogenesis is a multistep process taking years to develop from normal mucosa to malignancy, in this long period it is possible to intercept the process preventing its progression to an invasive stage. This strategy could be of special relevance in subjects at increased risk of CRC, such as patients with FAP or Lynch syndrome or patients with a personal history of CRC [16]. As a matter of fact, although some drugs (i.e., anti-inflammatory drugs) may exert preventive activity, their long-term use is hampered by side effects, pointing the need to discover new agents with chemopreventive activity that could be given alone or, perhaps, in combination using lower doses [16]. Of course, the product must be safe. Here we show that daily intake of BJe up to 70 mg/kg for 3 months displays no apparent signs of toxicity in Pirc rats. In this line, several studies performed in both animals and humans revealed that BJ or its polyphenol extracts did not exert any sign of systemic toxicity, suggesting their valuable efficacy and safety ratio [1, 37].

Numerous studies suggest that inflammation promotes carcinogenesis in various organs including the colon [38], and that natural products alone or in combination with approved anti-cancer therapies can be considered as a promising means of preventing and treating inflammation and cancer. In this line, it has been reported that agents with anti-inflammatory activity may lower carcinogenesis [39, 40]. To understand whether BJe may act through an anti-inflammatory effect, we studied the expression of some cytokines and enzymes expressed by tumour and/or infiltrating stromal cells, such as tumour-associated macrophages (TAMs), implicated in the pathogenesis of colorectal cancer in both humans and animal models [26]. Accordingly, TAMs (the M2-polarized TAM, particularly) have been indicated to promote tumorigenesis via the secretion of several cytokines [27]. Here we found that

the majority of the inflammatory markers, including those of TAM-M2, such as *Arginase 1*, *IL-6* and *IL-10* [41], were down-regulated in tumours from animals treated with BJe. Furthermore, we observed that other inflammatory markers such as *IL-1β*, also expressed in TAMs [42], as well as genes encoding for inflammatory enzymes (*iNOS* and *COX-2*), are down-regulated in the tumours from BJe-treated rats. Thus, the results pointed out a marked down-regulation of the inflammatory process in the tumours from BJe-treated animals, indicating an anti-inflammatory effect of BJe. This finding is in agreement with reports documenting anti-inflammatory and antioxidant effects of BJe in an experimental model of inflammatory bowel disease (IBD), associated with an increased risk of developing CRC [21].

Furthermore, in the tumour tissues from rats fed with 70 mg/kg of BJe, we also observed an increase of apoptosis, accompanied by the up-regulation of pro-apoptotic genes, among which *p53* expression particularly augmented in animals supplemented with the extract. This result agrees with previous studies [43, 44] showing increased expression of this gene in animals treated with putative chemopreventive agents. Given the role of *p53* in apoptosis, these results suggest that the chemopreventive activity of BJe may act through an increased ability of tumours to undergo apoptosis. Accordingly, in animals that daily assumed BJe, although not significantly, mRNA levels of *BAX* and *Bcl-2* were increased and reduced, respectively. We previously showed that *Survivin* is up-regulated in Pirc rats when compared with WT rats of the same age [15] as well as *p21*, an inhibitor of cell cycle progression, but also an anti-apoptotic factor [45]. In this study, we observed that the anti-apoptotic gene *Survivin* (alias *Birc 5*), as well as *p21*, were significantly down-regulated in animals fed with BJe, suggesting that the *Citrus* extract may normalize their expression, leading to an increased ability of tumours cells to undergo apoptosis. In agreement with this hypothesis, we microscopically observed that apoptotic cells, characterized by their altered morphology, are higher in tumours from BJe group than in those from controls. Our recent studies in Pirc rats treated with a pomegranate extract, indicate that substances endowed with chemopreventive activity act through an increase of apoptosis [25], suggesting that this is a general mechanism of action of anti-cancer drugs. Accordingly, it has been hypothesized that apoptosis may constitute a target for chemoprevention [46].

In conclusion, for the first time, we showed that BJe reduces spontaneous tumorigenesis in the colon of Pirc rats, through a mechanism involving, at least in part, its anti-inflammatory and pro-apoptotic activities. Thus, our results suggest the potential of this extract to inhibit carcinogenesis that could be exploited as a strategy to prevent CRC in patients at risk.

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Author contributions APF carried out the carcinogenesis and the histochemistry experiments, KT maintained the Pirc colony and, together with AR and CL, carried out part of the carcinogenesis experiment. GC determined apoptosis and proliferation in immunohistochemistry. SC and NF performed real-time PCR analyses. GC and MN conceived, designed and supervised the work, as well as drafted the manuscript, that was read and approved by all the authors.

Compliance with ethical standards

Conflict of interest The authors declared that are no conflicts of interest. Agrumaria Corleone provided BJe, but it had no other role in the study.

Ethical standards The animal studies have been approved by the Commission for Animal Experimentation of the Italian Ministry of Health (Authorization number 323/2016-PR) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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