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Pomegranate By-Products in Colorectal Cancer Chemoprevention: Effects in *Apc*-Mutated Pirc Rats and Mechanistic Studies In Vitro and Ex Vivo

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Scope: To investigate the effect of pomegranate mesocarp, a polyphenol-rich by-product of juice production, in colorectal cancer (CRC) chemoprevention.
Methods and results: A mesocarp decoction (PMD) is administered for 6 weeks in the diet to Pirc rats, mutated in *Apc*, a key-gene in CRC. Mucin-depleted foci (MDFs), as CRC biomarkers, are reduced in PMD-fed rats compared to controls (MDF/colon: 34 ± 4 versus 47 ± 3, $p = 0.02$). There is an increase in apoptosis in MDFs from PMD-treated rats compared to controls (2.5 ± 0.2 versus 1.6 ± 0.2, $p < 0.01$). To elucidate the involved mechanisms, two colon-relevant metabolites of the polyphenolic and fiber PMD components, urolithin-A (u-A) and sodium butyrate (SB), are tested alone or in combination in vitro (colon cancer cells), and ex vivo in adenoma (AD) and normal mucosa (NM) from Pirc rats. u-A 25 μM plus SB 2.5 mM (USB) causes a significant reduction in COX-2 protein expression compared to untreated controls (about –70% in cancer cell cultures, AD, and NM), and a strong increase in C-CASP-3 expression in cells (about ten times), in AD and NM (+74 and +69%).
Conclusion: These data indicate a chemopreventive activity of PMD due, at least in part, to pro-apoptotic and anti-inflammatory action of its metabolites that could be exploited in high-risk patients.

1. Introduction

Considering the significant impact of cancer in terms of human lives and economic cost (US$ 1.16 trillion in 2010, World Cancer Report 2014, http://www.who.int/mediacentre/factsheets/fs297/en/), there is a continuous interest in searching new mechanisms to be targeted, and in implementing preventive strategies such as chemoprevention, that is "the use of natural, synthetic, or biologic agents, able to delay, reverse, or inhibit tumor progression".[1] Colorectal cancer (CRC), the second leading cause of cancer death in Europe (IARC, GLOBOCAN 2012 http://gco.iarc.fr/today/home), develops through a sequential multistep progression of epithelial cells initiated to a cancerous state with defined precancerous intermediaries. Numerous trials document the ability of nonsteroidal anti-inflammatory drugs (NSAIDs) to prevent CRC, but the chronic use of these drugs increases the risk of serious cardiovascular events, so that alternative strategies are needed.[2,3] In the last thirty years, several studies focused on the effects of polyphenols from various sources (i.e. curcumin, resveratrol, and green tea) on colorectal carcinogenesis in vitro, in vivo, and in some clinical trials, testing their synergistic effects in association with chemotherapeutic treatments or their preventive activity in subjects at high CRC risk[4–6] (https://clinicaltrials.gov/). Among fruits, with a high polyphenolic content, pomegranate (*Punica granatum)* is endowed with antioxidative, anti-inflammatory, and anticancer activities.[7–9] The predominant phenolic component of pomegranate belongs to the ellagitannin family (ETs), among which punicalagin, showing pronounced antiproliferative and anti-inflammatory activities in addition to antioxidative effects, is peculiar of this fruit.[10–12] Several studies reported that total phenolic compounds (TPC) and punicalagin are more abundant in pomegranate mesocarp and peel (pericarp) than in arils and seeds, the latter being used for industrial juice production.[13] Beside polyphenols, the polysaccharide component, also present in mesocarp, has been suggested to exert anticancer activity, at least in in vitro systems.[14,15] Regarding the anticancer activity in vivo, two studies investigated in rats the effect of commercial pomegranate juice preparations on the induction of aberrant crypt foci (ACF), purported preneoplastic lesions and showed that the juice reduces the number of these lesions.[16,17] Besides the juice, other parts of pomegranate such as seed oil and peel have been reported to reduce chemically induced colon carcinogenesis in rats.[18–20] Similarly, Sadik and Shaker[21] reported that a commercial phytochemical extract of pomegranate might indeed have

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chemopreventive activity. Instead, the effect of mesocarp has not been investigated.

Taking into account these studies and the recent encouragement to the implementation of new approaches for the efficient use of the huge biomass into a spectrum of bio-based products, a concept named "biorefining" (Sustainable development goals 09/2015, UN; Bioenergy task 42, IEA. https://sustainabledevelopment.un.org/?menu=1300, http://www.iea-bioenergy.task42-biorefineries.com/en/ieabiorefinery.htm), we thought it of interest to investigate the possibility of employing a pomegranate mesocarp decoction (PMD), characterized in polysaccharide and ellagitannin content, for CRC prevention. After its characterization, PMD was tested in vivo in a genetic model of CRC, the Pirc rat. This strain bears an heterozygote mutation in the *Apc* gene, the key genetic event in colorectal carcinogenesis.[22] Due to this mutation, Pirc rats spontaneously develop colon polyps, thus mimicking both familial adenomatous polyposis (FAP) and sporadic colon cancer.[6,22,23] Pirc rats also present microscopic preneoplastic lesions in the colon, called mucin depleted foci (MDF), which represent the early step in the development of CRC and can thus be used as tumor biomarkers in chemoprevention studies.[23,24] In addition to the chemopreventive experiment in vivo, to investigate the mechanisms involved in the effects observed at the level of early carcinogenesis phases, and to assess the capacity of PMD to influence carcinogenesis at more advanced phases, we measured the activity of two main metabolites of PMD: urolithin-A (u-A) and sodium butyrate (SB), in vitro on HT-29 and HCT-116 colon cancer cells and ex vivo in biopsies of normal colon mucosa (NM) and colon adenomas (AD) from Pirc rats. u-A is the main punicalagin metabolite in the colon[25,26]; SB, one of the main short chain fatty acid produced by fermentation of the soluble fiber component in the colon, has anticancer activity in vitro and in vivo.[27,28]

2. Experimental Section

2.1 Chemical reagents

Gentamicin, Hank's Balanced Salt Solution (HBSS), DTT, thiazolyl blue tetrazolium bromide (MTT), RIPA buffer, and protease/phosphatase inhibitors were from Sigma–Aldrich (Milan, Italy). $\alpha + \beta$ punicalagins and ellagic acid were from Merck. AIN76 diet components were from Piccioni (Milan, Italy). NucleoSpin® TriPrep kit for RNA extraction was from Machery-Nagel GmbH & Co. KG (Duren, Germany). Cell culture materials were from GIBCO, BRL (Rodano-Milan, Italy). u-A was from Toronto Research Chemicals (Toronto, Canada). SB was from Merck-Millipore (Vimodrone-Milan, Italy). MTS solution was from Promega (Gessate, Italy). Protein assay was performed using DC Protein Assay kit from Bio-Rad (Segrate-Milan, Italy).

2.2. Pomegranate Mesocarp Decoction Preparation and Characterization

Pomegranate ripe fruits of Wonderful variety cultivated in Puglia, Italy (harvested in 2015), were used to recover the mesocarp. Eighteen kilograms of fresh fruits were manually treated to separate the mesocarp from the other parts of the fruit. The mesocarp (80% moisture) was then used to prepare a decoction through boiling in water for 1 h (extractive ratio 1:40 w dried mesocarp/v). The sample was cooled, filtered, and the solution freeze-dried, then ground and used as powder (PMD). The final yield was 75% of dried mesocarp.

The total fat content was extracted by Soxhlet, and gravimetrically determined according to ISS protocol (1996/34); the protein content (PC) was evaluated by Kjeldhal method: PC (g/100 g) = $N \times 6.25$ (N = total nitrogen. Lastly, soluble and insoluble dietary fiber analysis was carried out according to AOAC method 991.43 (Determination of soluble, insoluble, and total dietary fiber in foods and food products, final approval 1991).

The HPLC–DAD analysis was carried out using a Sinergi Fusion 150 mm × 2 mm id, 4 μm, RP-18, column from Phenomenex (Bologna, Italy), with a flow rate of 0.2 mL min^{-1}. The mobile phase was constituted by A, acidified water by HCOOH (0.1% v/v) and B, acetonitrile. The linear multistep solvent gradient started from 95% A and reached 75% A in 4 min, followed by a 4-min plateau; the next step was 6 min to reach 65% A, and finally 2 min to reach 10% A and 90% B, with a reequilibration time of 10 min and a total time of analysis of 26 min. The ellagitannins determination was performed using as external standards $\alpha + \beta$-punicalagins (at 380 nm, linearity range between 0.5–8 μg) and ellagic acid (at 370 nm, linearity range of 0.031–1.25 μg) obtaining calibration curves with R^2 0.998 and R^2 0.9995, respectively.

2.3. 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.[29] Male Pirc rats, aged 4 weeks, were randomly assigned to: controls ($n = 10$), fed with standard AIN-76 diet, or PMD-treated ($n = 11$) fed with the same AIN-76 diet supplemented with 10 000 ppm of PMD, corresponding, on the basis of the polyphenolic content of PMD, to a dose of 50 mg kg^{-1}/die of total polyphenolic compounds. Rats were sacrificed by CO_2 asphyxia after 6 weeks of treatment, in line with the experimental protocol approved by the Commission for Animal Experimentation of the Italian Ministry of Health (Authorization number 323/2016-PR).

2.4. Processing of Colon, Sample Collection, and Determination of Mucin Depleted Foci

At sacrifice, the entire intestine was dissected, flushed with cold saline, and the apparently Normal Mucosa (NM) from the proximal portion of the colon was scraped and stored at –80 °C in RNAlater™ (RNA stabilization Reagent, Qiagen) as reported.[29] A small sample of NM from the medial portion (about 9 mm^2) was collected and fixed in 10% formalin solution to assess proliferative and apoptotic activity. The remaining colon and rectum were fixed in formalin and stained with high-iron diamine Alcian blue (HID-AB). This technique highlights mucin production and allows, with a topographical observation of the unsectioned colon, the determination of the number of MDF (number of MDF/colon) and their multiplicity (number of crypts forming each MDF).[23] After the enumeration of MDFs, these were

marked with permanent ink, dissected under microscope and then embedded in paraffin in such a way that crypts could be sectioned longitudinally (4 μm thick) to determine apoptosis, see below.[29]

2.5. Cell Proliferation and Apoptosis in NM and MDF

Proliferative activity was assessed as previously reported[29] in the morphologically NM, determining proliferating cell nuclear antigen (PCNA) immunoreactivity with a mouse monoclonal antibody (PC-10, Santa Cruz, CA, USA) at 1:1000 dilution. Proliferative activity was expressed as labeling index (LI): number of cells positive to PCNA/cells scored × 100, evaluated in at least 15 full longitudinally sectioned crypts of the NM. Histological sections of the NM (4 μm thick) were also stained with hematoxylin-eosin to determine the number of apoptotic cells in at least 15 full entire longitudinally sectioned crypts.[29] Apoptosis was also evaluated in histological sections of MDFs dissected as previously described.[30]

2.6. Semi-Quantitative RT-PCR

Gene expression was evaluated in the NM, taken at the sacrifice as described above. Total RNA extraction, reverse-transcription of 1 μg of total RNA and subsequent PCRs were performed as previously described.[31] For each target gene, the relative amount of mRNA in the samples was calculated as the ratio of each gene to β-actin mRNA (primers used are shown in **Table 1**).

2.7. Western Blotting

Protein expression was evaluated in samples from in vitro, in vivo, and ex vivo experiments. For cultured cell lines, 40 μL of RIPA-buffer supplemented with 1% protease inhibitors and 1% phosphatase inhibitors were added to each well, and the obtained protein solution was sonicated for 15 sec and centrifuged for 1 min at 14 000 rpm and 4 °C. For colon samples, scraped NM in the in vivo experiments and ex vivo samples of AD and NM were homogenized in RIPA-buffer in the proportion of 8 μL mg^{-1} tissue for no more than 2 min, sonicated, and centrifuged as above. Each supernatant was collected and the PC was measured. For Western blotting, 40 μg of protein extracts were used for each experimental point. Electrophoretic running, immunostaining, band acquisition, and quantification were performed as previously described.[32] Each measured density was normalized by using the corresponding GAPDH density value.

The antibodies used were: COX-2 (160126 rabbit, Cayman Chemical), 1:200; PCNA (PC10: sc-56 mouse Santa Cruz Biotechnology, INC), 1:1000; C-CASP-3 (Asp175 Rabbit Cell Signaling), 1:1000; NOS-2 (N-20 rabbit Santa Cruz Biotechnology, INC) 1:500; BAK (rabbit Anti-BAK, NT Millipore) 1:500; GAPDH (14C10 Rabbit mAb Cell Signaling) 1:3000; anti-rabbit IgG antibody (Cell Signaling), 1:4000, and anti-mouse IgG (Chemicon, Temecula, CA), 1:5000.

Table 1. Primers used for the amplification of different genes by RT-PCRs.

Gene	Forward	Reverse
β-actin	5′-ACCACAGCTGAGAGGGAAAT-3′	5′-AGAGGTCTTTACGGATGTCAAC-3′
IL-6	5′-TCTCTCCGCAAGAGACTTCC-3′	5′-TCTTGGTCCTTAGCCACTCC-3′
IL-1β	5′-TGACCCATGTGAGCTGAAAG-3′	5′-AACTATGTCCCGACCATTGC-3′
S100A9	5′-GCACGAGCTCCTTAGCTTTG-3′	5′-GACTTGGTTGGGCAGATGTT-3′
NOS-2	5′-GCCTAGTCAACTACAAGCCCC-3′	5′-CCTGGGGTTTTCTCCACGTT-3′
COX-2	5′-ACGTGTTGACGTCCAGATCA-3′	5′-GGCCCTGGTGTAGTAGGAGA-3′

2.8. Cells Cultures and Treatments

HT-29 and HCT-116 cell lines (provided from ATCC) were grown in high glucose Dulbecco's Modified Eagle's Medium/RPMI1640 Medium respectively, both supplemented with 10% of fetal bovine serum, PenStrep (penicillin 100 U mL^{-1}, and streptomycin 0.1 mg mL^{-1}), maintained at 37 °C in a cell incubator (5% CO_2), and used during the linear phase of growth. For experimental treatments, cells were plated in 12 MW (130 000 cells/well) and treated starting on the following day with standard medium or: (a) u-A (3,8-diydroxybenzo(c)chromen-6-one, u-A) 0.1, 1, 10, 25, 50, and 100 μM; (b) SB 50, 100, 500 μM, 1, 5, and 10 mM; (c) u-A 25 or 50 μM + SB 500 μM, 1, and 5 mM (USB). Treatments lasted 24 or 72 h.

2.9. MTS Viability Assay in Cell Cultures

Cell proliferation assays were performed in 96 MW (8000 cells/well) after 72 h treatments. At the end of this time, media were removed, cell monolayers were washed two times with 1× PBS, and 100 μL/well of DMEM or RPMI with 5% FBS and 20 μL of ready-to-use MTS solution were added. The measurement of absorbance at 490 nm was performed after 90-min incubation at 37 °C in 5% CO_2 humidified atmosphere.

2.10. Ex Vivo Short-Term Cultures and Treatment

Pirc rats (n = 4) at 8 months of age were sacrificed as described above. The colon was rinsed two times with PBS supplemented with Pen-Strep (penicillin 100 U mL^{-1} and streptomycin 0.1 mg mL^{-1}) and gentamycin (50 μg mL^{-1}), then opened to collect samples of AD and apparently NM. All samples were transferred in 1.5 mL tubes containing 40 mM DTT in HBSS, rinsed with HBSS supplemented with Pen-Strep and gentamycin 50 μg mL^{-1} (HBSS/PSG), and dissected under microscope. ADs and NMs were dissected into approximately equal parts (weight range: 10–15 mg). Samples were then transferred in 48 MW containing DMEM (control) or DMEM containing u-A 25 μM + SB 2.5 mM (USB) and maintained for 24 h in cell incubator at 37 °C and 5% CO_2. All these procedures were performed within 1 h from sacrifice.

Cell viability of AD and NM samples was assessed in separate samples performing the MTT test at time zero (T_0) and

after 24 h (T_{24}). Briefly, samples dissected under microscope were transferred in 2 mL tubes containing HBSS/PSG. Then, T_0 samples were transferred in 48 MW containing 200 μL of DMEM without red-phenol supplemented with 1% FBS, Pen-Strep, 50 μg mL^{-1} gentamycin and 1 mg mL^{-1} MTT and incubated for 3 h at 37 °C in cell incubator. At the end of the incubation, samples were transferred in 2 mL tubes containing acidified isopropanol (isopropanol + HCl 4 mM and 0.1% Nonidet-P40), weighted, and incubated at 37 °C with shaking for 3 h to extract MTT. Subsequently, they were centrifuged for 5 min at 4 °C and 1000 rpm. The T_{24} samples after dissection were transferred into 48 MW containing DMEM, incubated for 24 h and then subjected to the MTT test as described for the T_0 counterparts. For all samples, 200 μL of each supernatant were then transferred in 96 MW for measuring MTT absorbance at 570 nm in a plate reader spectrophotometer. All measures were performed in duplicate and the absorbance values (A) were normalized to the weight of the sample after MTT incubation (A mg^{-1}).[33] Finally, the viability at 24 h was expressed as percentage (%) of the corresponding T_0 counterpart.

2.11. Statistics

Differences in MDFs, immunological and morphological indexes and RT-PCR data between PMD group and controls were analyzed with the *t*-test for unpaired samples. Data from MTS test and Western blotting assay in vitro were analyzed by one-way ANOVA followed by Bonferroni's multiple range test, with GraphPad Prism 5.0 (GraphPad Software) as appropriate. Western blotting data from the ex vivo experiments were subjected to two-way ANOVA to take into account the effect of both treatment and tissue type (AD or NM).

3. Results

3.1. Composition of PMD

The proximate composition of the dried decoction resulted to be: proteins 1.9%, total sugar 0.4%, dietary fibers about 11% (soluble 10.9%, insoluble < 1%), and fat was absent, as expected. The decoction contained also ellagitannins (151.47 ± 3.42 mg g^{-1}) with a prevalence of $\alpha + \beta$ punicalagins (67.5 ± 1.24 mg g^{-1}) and a minor content of ellagic acid and derivatives (21.7 ± 0.35 mg g^{-1}).

3.2. Effect of PMD on Colon Tumorigenesis in Pirc Rats

The mean weight of the rats at the beginning of the treatment (4 weeks of age) was 60 ± 3 g (means ± SE, $n = 21$). At sacrifice, when the animals were 10 week old, the mean weight was similar between controls and PMD group (216 ± 11 g in controls ($n = 10$) versus 217 ± 6 g in PMD treated ($n = 11$), means ± SE), with no apparent sign of toxicity of the treatment.

The number of the preneoplastic lesions MDFs was significantly reduced ($p = 0.02$) in PMD-treated rats compared to controls (**Figure 1**, panel A; Supporting Information, Figure S1). Moreover, PMD-treated rats showed MDF with a significantly lower multiplicity (i.e. the lesions were formed by a lower number of crypts) when compared to those in the control rats ($p = 0.03$) (Figure 1, panel B). Accordingly, measuring apoptosis in these lesions, we found a significant increase in apoptotic index in the MDFs dissected from PMD-treated rats (Figure 1, panel C).

3.3. Proliferation, Apoptosis, and Inflammation in the Normal Colon Mucosa of Pirc Rats

To understand the molecular mechanisms of action underlying this protective effect of PMD, we determined the gene expression of inflammatory proteins, apoptosis, and proliferation in the NM of both groups. Among the inflammatory genes, we measured by RT-PCR the mRNA expression of *S100-A9, IL-6, IL-1β, NOS-2* and *COX-2* in NM samples. The results showed that none of these genes was influenced by PMD administration (data not shown). Protein levels evaluation (Western blot) of NOS and COX-2 confirmed the gene expression data, indicating no difference between control and treated animals (data not shown).

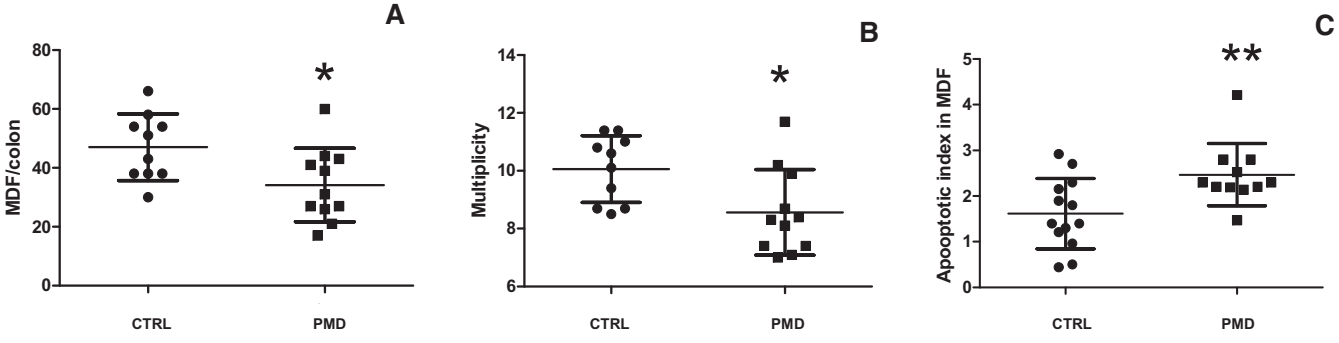

Figure 1. Pre-neoplastic lesions: MDF/colon (panel A), MDF multiplicity (number of crypts/MDF (panel B) and apoptotic index in MDF (panel C) in Pirc rats fed with AIN76 (CTRL) or AIN76 diet supplemented with 50mg kg^{-1}/die of PMD (means ± SD); controls: $n = 10$, PMD-treated: $n = 11$. Each point represents data from a single animal, means ± SD for each group are also shown. *, **: significantly different from controls (Student *t*-test), $p < 0.05$ and $p < 0.01$, respectively.

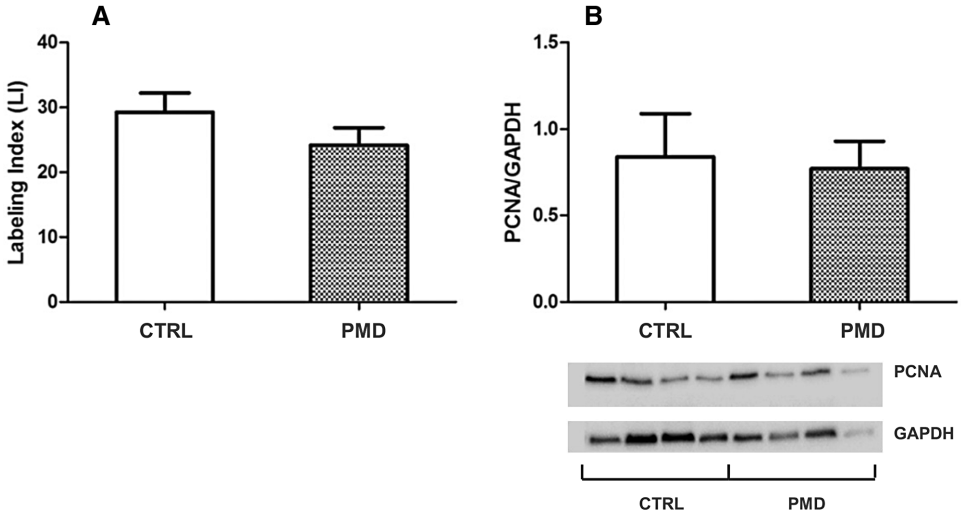

Figure 2. Effects of PMD in the Pirc normal colon mucosa: proliferation activity evaluated as LI in histological sections immunostained with a PCNA-antibody, and PCNA protein expression by Western blot (panels A and B respectively) in NM of Pirc rats treated with PMD. Bars are means + SE; controls: $n = 10$, PMD-treated: $n = 11$. Examples of Western blotting membranes showing bands relative to single animals are shown below the respective graph.

Concerning the impact on proliferation, the LI in colon sections immunostained with PCNA, as well as PCNA immunoblotting in NM samples, showed that proliferative activity was not affected by PMD (**Figure** 2, panels A and B). Apoptosis determined in histological sections of NM based on nuclear morphology was also similar in the two groups (AI: 0.15 ± 0.03 and 0.21 ± 0.11 in controls and treated animals, respectively, means ± SE). In agreement with the histological analysis, Western blot determination of C-CASP-3 and BAK proteins showed no statistically significant difference between control and treated groups (CASP-3/GAPDH: 8.93 ± 1.9 versus 10.07 ± 2.02; BAK/GAPDH: 0.51 ± 0.21 versus 1.10 ± 0.29 in controls ($n = 10$) and in PMD treated ($n = 11$) groups respectively, means ± SE).

3.4. Effect of Pomegranate Metabolites on Colon Cancer Cells Viability

Based on the protective effects observed in vivo on microscopic preneoplastic lesions (MDFs), we also evaluated the effects of pomegranate products in more advanced steps of colon carcinogenesis, such as human colon cancer cell lines and macroscopic ADs from older Pirc rats. For these in vitro and ex vivo experiments, two main colon-relevant metabolites of the polyphenolic and fiber PMD components, u-A and SB, were used. First, we investigated the effect of different doses of u-A and SB on the viability of HT-29 cells upon 72 h exposure (**Figure 3**, panels A and B). The IC_{50} for u-A resulted to be 43.9 μM, and that of SB 3 mM. The combination of u-A and SB was also tested (Figure 3 panel B, dotted line): in the presence of a fixed concentration of u-A (25 μM, inducing a 25% reduction in cell viability) the IC_{50} of SB was not statistically different from that of SB alone, indicating the absence of a synergistic effect. However, at SB concentrations lower than IC_{50}, the copresence of u-A further reduced viability, (Figure 3 panel B, compare dotted and continue lines), suggesting an additive type interaction between the two metabolites. Similar results were found in HCT-116 human colon cancer cell line (IC_{50} for u-A 59.2 μM, for SB 0.7 mM). These data are shown in Supporting Information, Figure S2 (panels A–B).

3.5. Proliferation, Apoptosis, and Inflammation in Colon Cancer Cells Treated with Urolithin-A, Sodium Butyrate, or Both

The effect of u-A and SB on markers of proliferation, apoptosis, and inflammation was assessed in HT-29 cells by means of Western blotting. Cells were treated for 24 or 72 h with single compounds at concentrations below the respective IC_{50}s measured in the cell viability experiments, or with a combination of the two metabolites at concentrations giving additive effects on cell viability reduction: for HT-29, u-A 25 μM, SB 2.5 mM, and u-A 25 μM + SB 2.5 mM (USB); for HCT-116, u-A 50 μM, SB 0.5 mM, u-A 50 μM and SB 0.5 mM (USB). In HT-29 cells, the expression of the proliferation marker PCNA was significantly reduced by SB after 24 h, while u-A and USB reduced it slightly, but not significantly, compared to the respective controls. At 72 h, the untreated cells showed a slight reduction in proliferation compared to 24 h, and the USB combination brought about a further significant decrease compared to respective 72 h control (Figure 3, panel C). As for apoptosis, activated Caspase-3 (C-CASP3) expression was strongly increased by the combination of u-A and SB by 24 h; during the following 48 h, the levels in treated cells were still higher compared to the respective controls, although the differences were no longer significant (Figure 3, panel D). Interestingly, we also observed that in cells treated for 72 h, the expression of the inflammatory markers iNOS and COX-2 was reduced by all the treatments (Figure 3, panels E and F respectively), with the USB treatment being the most effective (–79.7 and –74.5% compared to respective controls for iNOS and COX-2 respectively, $p < 0.01$). Notably, in the

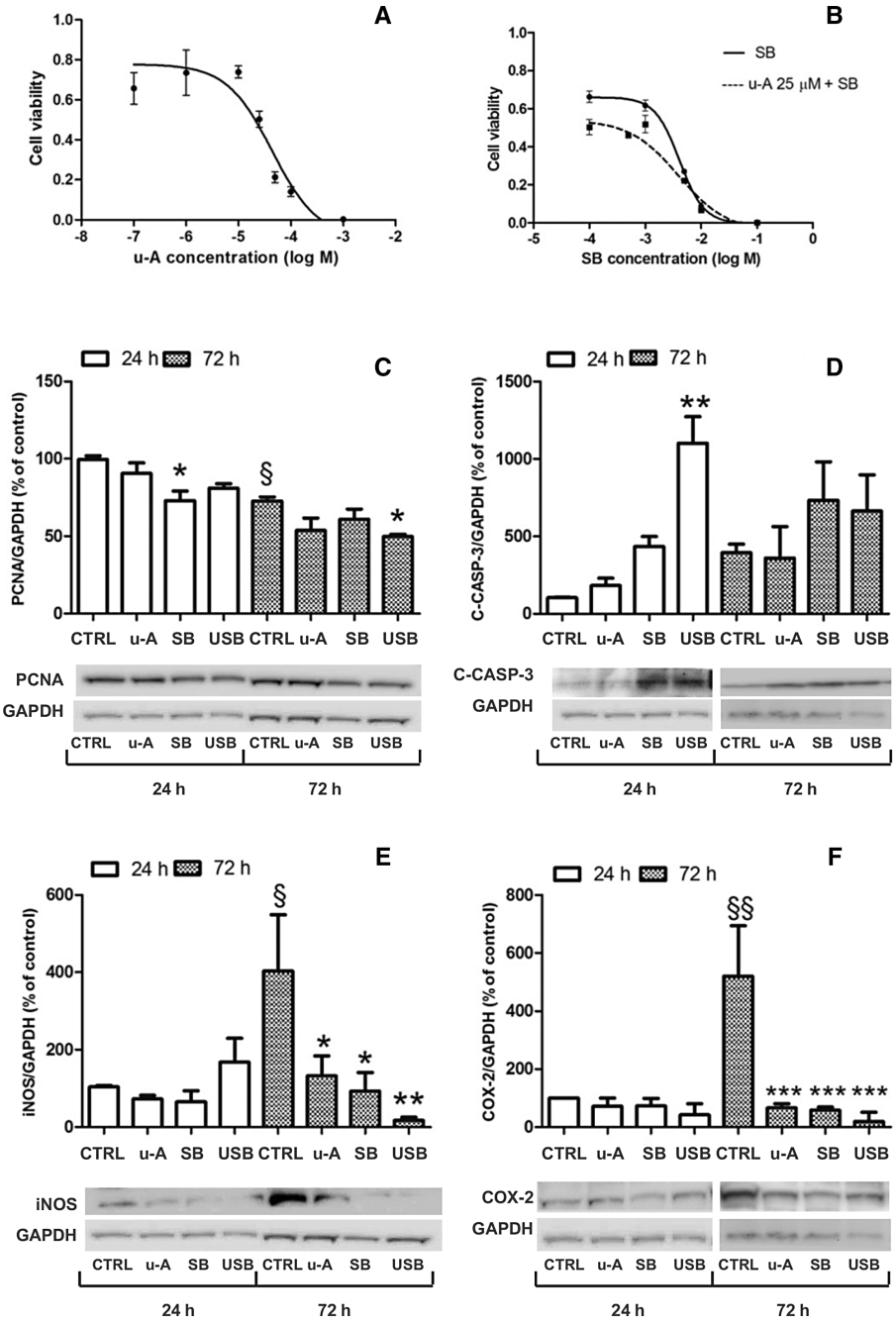

Figure 3. Effects of pomegranate metabolites on HT-29 cells: panel A: concentration-response curve of u-A (log IC_{50} = –4.358, R^2 = 0.8274); panel B: concentration-response curves of SB (logIC_{50} = –2.511, R^2 = 0.9224) and SB plus u-A 25 μM (dotted line, logIC_{50} = –2.405, R^2 = 0.9275); incubation time: 72 h. Panels C–F: relative protein expression of PCNA, C-CASP-3, iNOS, and COX-2 respectively, after 24 and 72 h treatment with u-A 25 μM (u-A), SB 2.5 mM (SB) or u-A 25 μM plus SB 2.5 mM (USB) compared to the respective control (CTRL). Bars are means + SE of four experiments; *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ significantly different from respective controls. §: $p < 0.05$, §§: $p < 0.01$ significantly different from 24 h control (one-way ANOVA). Examples of Western blotting membranes are shown below the respective graph.

untreated 72 h controls, iNOS and COX-2 protein levels were higher compared to 24 h controls; no significant effects of the treatments were observed at 24 h. Similar results were obtained with HCT-116 cells (Supporting Information, Figure S1, panels C–F).

3.6. Effect of Pomegranate Metabolites on Proliferation, Apoptosis, and Inflammation in Biopsies from Pirc Adenomas and Normal Mucosa

Colon AD and NM samples from Pirc rats were used for short-term (24 h) ex vivo experiments, in which the combination of

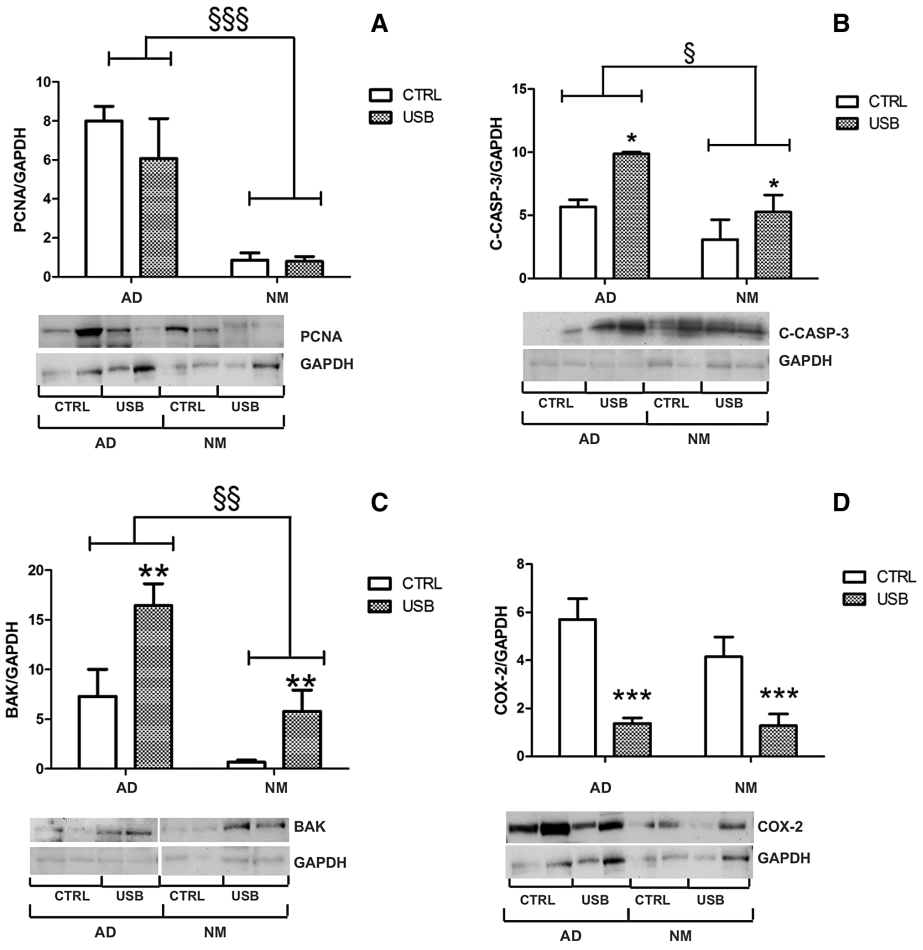

Figure 4. Effects of pomegranate metabolites in Pirc ADs and NM biopsies ex vivo: relative protein expression of PCNA, C-CASP-3, BAK, and COX-2 (panels A–D, respectively) in control (CTRL) and USB-treated AD and NM samples after 24 h in culture. Bars are means + SE of four experiments; §, §§, and §§§: $p < 0.05$, <0.01, and < 0.001, respectively, comparing AD versus NM (two way ANOVA). *, **, and ***: $p < 0.05$, < 0.01, and < 0.001, respectively, comparing treated versus untreated samples (two-way ANOVA). Examples of Western blotting membranes are shown below the respective graph: two different experimental points are present for each condition.

u-A and SB treatment (USB), being the most effective in the in vitro experiments, was tested at the same concentration used in HT-29 cells. Viability of the samples, assessed with the MTT method at time 0 (control) and after 24 h of incubation, was 75 and 50% of the corresponding values at 0 time for NM and AD, respectively.

The effect of USB treatment on proliferation, evaluated by means of PCNA Western blot, was slight and nonsignificant both in AD and in NM samples (**Figure 4**, panel A). On the contrary, USB showed a prominent proapoptotic effect in AD, as indicated by increased C-CASP-3 and BAK expression (Figure 4, panels B and C, respectively) compared to untreated controls; a significant increase in these two proapoptotic proteins was also observed in the NM. Finally, a marked anti-inflammatory effect on both AD and NM samples treated with USB was observed (Figure 4, panel D): COX-2 protein expression was decreased by about 77% in AD and 69% in NM. Interestingly, the two-way ANOVA analysis on these data showed that, as expected, basal proliferative activity and apoptosis level were higher in the AD tissue compared to the NM.

4. Discussion

The majority of the published studies on chemopreventive effects of pomegranate focused on the juice obtained either from whole fruits or arils.[34] Despite the fact that peel and mesocarp also contain polyphenols, to which the beneficial effects of pomegranate are ascribed,[10,35] only few studies investigated the effects of peel[18,19] and no studies, to our knowledge, focused on mesocarp. In the present study, we evaluated the possibility of employing a decoction obtained from pomegranate mesocarp (PMD), a juice production by-product, as a source of molecules with chemopreventive activity in CRC. The decoction we used was characterized by a high content in ellagitannins (about 15% w/w), and a moderate content in soluble fibers, providing a manageable powder with low hygroscopicity. Fibers from different vegetables can act as a vehicle for polyphenols in the colon improving their bioavailability, particularly after the fermentation of the substrate.[36] Interestingly, we recently demonstrated that the polysaccharide component of Wonderful pomegranate has prebiotic properties in vitro,[37] and our preliminary experiments

on the advanced M-Shime® gastro-simulator also pointed out a beneficial effect of PMD, mainly in terms of short chain fatty acids production by the human microbiota (manuscript in preparation), suggesting prebiotic properties which have been associated with beneficial effects against colon carcinogenesis.[38] PMD was first tested in vivo, at a dose corresponding to a daily intake of 50 mg kg^{-1} of polyphenols, on Pirc rats. The Pirc rat, with a germ line mutation in the *Apc* gene, is a robust model for CRC chemopreventive studies,[6] as it spontaneously develops colon ADs and, at young age, microscopic preneoplastic lesions (MDFs) that can be used as an end-point in short-term chemopreventive studies.[39] Importantly, at variance with other genetic models such as Min mice, developing spontaneous tumors mostly in the small intestine and not in the colon, Pirc rats allow to study colon carcinogenesis and its chemoprevention in the very same environment, including the luminal content, in which the human disease develops. Our results clearly show that both the number of MDFs and their size in terms of crypts forming each MDF (multiplicity) were significantly reduced by PMD treatment, suggesting that indeed PMD is able to reduce colorectal tumorigenesis. Considering the number of MDF, the effect of PMD amounts to about 30% inhibition, a figure similar to that observed in rodents subjected to known chemopreventive treatments such as low fat diets, calcium, or aspirin.[40,41] We also observed that the level of apoptosis was significantly higher in MDFs from the PMD group than in MDFs from controls, suggesting that the observed protective effect may be due to increased apoptosis in these lesions, leading to elimination of precancerous cells. Increased apoptosis in tumors and preneoplastic lesions was previously observed in animals treated with compounds showing preventive activity.[42] In the NM of PMD-treated rats, only slight effects on proliferation, apoptosis, and inflammation were observed compared to controls.

To elucidate the molecular mechanisms involved in the protective effects observed in vivo, and to verify the efficacy of PMD treatment at more advanced carcinogenesis phases (i.e. in cancer cell lines and in macroscopic ADs), we tested the effect of two colon-relevant metabolites of the polyphenolic and fiber components of PMD: u-A and SB, respectively.[25,28] These were assessed in vitro on HT-29 human colon cancer cell line, bearing an *Apc* gene mutation, and on HCT-116 human colon cancer cell line, bearing a wild-type *Apc*. u-A and SB alone demonstrated inhibitory effects on cell viability with IC_{50} values in line with those reported previously.[8,43] We also evaluated the effect of the combination of these two metabolites, and found an additive effect on cell viability reduction of u-A and SB. We then focused on the combination of u-A and SB (USB), choosing concentrations of the two metabolites inducing per se less than 40% reduction in cell viability. Western blotting analyses of several protein markers involved in proliferative, inflammatory, and apoptotic mechanisms showed that USB treatment was capable of affecting these parameters in a more pronounced manner than single treatments in both cell lines. In fact, USB treatment was more effective on proliferative activity (PCNA reduction) and on apoptosis induction (C-CASP-3 increase). Besides, although u-A and SB alone were both able to lower iNOS and COX-2 after 72 h treatment, in line with previously reported data,[44,45] the anti-inflammatory effects of the combination were again more pronounced. The efficacy demonstrated by USB in cancer cell lines encouraged us to verify the efficacy of the combined treatment on macroscopic ADs of Pirc rats. For this purpose, and to reduce the number of experimental animals, we performed ex vivo experiments, in which small samples of ADs and NMs of 8-month-old Pirc rats were collected and maintained in culture for 24 h with or without USB at the same concentrations tested in HT-29 cells. In the control samples, the MTT method showed that the tissue was still viable at 24 h. Furthermore, PCNA expression (proliferative activity) and cleaved CASP-3 and BAK expression (apoptosis) were significantly higher in AD compared to NM, in agreement with many studies[29,46] documenting higher proliferation and apoptosis in AD compared to NM. Thus, these results indicate that this ex-vivo system is quite reliable. The treatment with USB increased apoptosis, as measured by BAK and C-CASP-3 protein level, in both AD and NM, compared to untreated controls. A proapoptotic effect of PMD was also observed in vivo in MDFs from PMD-treated rats, while in the NM in vivo this effect was not detected. A significant reduction in COX-2 expression was brought about by USB in ex vivo-treated AD and NM samples; regarding the NM, again this result is at variance with the in vivo studies were COX-2 expression was not varied. The discrepancies observed between the in vivo and the ex vivo results for apoptosis and COX-2 in NM might be due to modifications associated with the incubation in an oxygen-rich environment, which might sensitize cells to apoptosis and shift them towards an inflammatory phenotype. Thus, the expression of COX-2 in the NM ex-vivo might be higher than in physiological conditions and a reduction by PMD could be more easily observed. Finally, regarding proliferation, a slight effect of USB was observed on AD, while PCNA expression in NM was not affected by USB treatment, in agreement with what observed in the in vivo experiments. The concentrations of butyrate that we have used in the in vitro and ex vivo experiments are attainable in the colon in vivo.[26,28] For u-A, Nunez Sanchez et al.[4] demonstrated that in colon tissues of human subjects treated with a pomegranate peel extract for 15 days, u-A levels were around 850 ng g^{-1} of tissue, roughly corresponding to 4 μM concentration within the tissue. Thus, an extracellular concentration of 25 μM, as we have used in the present experiments, appears to be attainable.

5. Concluding Remarks

This is the first study to test the chemopreventive activity of pomegranate mesocarp in the Pirc rat, a relevant genetic model of colon carcinogenesis. Furthermore, we describe for the first time the combined effects of u-A and butyrate in vitro and the effect of this metabolite combination on ADs ex vivo.

As a whole, this work indicates that pomegranate mesocarp-based products have the potential to counteract the very initial, intermediate, and late stages of carcinogenesis, being potentially useful both in colon cancer primary and secondary prevention. The mechanism of action involves an increase in apoptosis in the preneoplastic lesions MDFs. Parallel in vitro and ex vivo experiments indicate that the chemopreventive effect of PMD may be due, at least in part, to the proapoptotic and anti-inflammatory effects of its colon metabolites u-A and SB, and especially to their combination.

An added value to the use of by-products of juice production is the fact that PMD was obtained by a green simple process. Furthermore, the particular combination of ellagitannins (up to 15%) and the pool of fermentable polysaccharides (about 10%) of PMD may increase the production of bioactive metabolites, such as urolithins and butyrate, in the colon. Thus, this study underlines the potential value of these by-products as a source of bioactive molecules that could be exploited for CRC prevention in high-risk subjects.

Abbreviations

AD, adenoma; CRC, colorectal cancer; MDF, Mucin Depleted Foci; NM, normal mucosa; PMD, Pomegranate Mesocarp Decoction; SB, sodium butyrate; u-A, urolithin-A

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgments

KT carried out the in vitro and ex vivo experiments and drafted the manuscript together with LG and GC. APF carried out the carcinogenesis experiment and gene expression analysis. AR and IS carried out part of the carcinogenesis experiments and the gene expression and histochemistry experiments. MK prepared the decoction and carried out the relative chemical analyses together with NM. LG performed the in vitro experiments. GC determined apoptosis and proliferation with immunohistochemistry. GC, LG, and NM conceived, designed, and supervised the work. All authors have read and approved the manuscript.

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Conflict of Interest

The authors have declared no conflict of interest.

Keywords

butyrate, colon carcinogenesis, mucin depleted foci, pomegranate mesocarp, urolithin

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[1] M. B. Sporn, D. L. Newton, *Fed. Proc.* **1979**, *38*, 2528.
[2] S. D. Solomon, J. J. McMurray V, M. A. Pfeffer, J. Wittes, R. Fowler, P. Finn, W. F. Anderson, A. Zauber, E. Hawk, M. Bertagnolli, *New Engl. J. Med.* **2005**, *352*, 1071.
[3] G. Steinbach, P. M. Lynch, R. K. Phillips, M. H. Wallace, E. Hawk, G. B. Gordon, N. Wakabayashi, B. Saunders, Y. Shen, T. Fujimura, L. K. Su, B. Levin, L. Godio, S. Patterson, M. A. Rodriguez-Bigas, S. L. Jester, K. L. King, M. Schumacher, J. Abbruzzese, R. N. DuBois, W. N. Hittelman, S. Zimmerman, J. W. Sherman, G. Kelloff, *New Engl. J. Med.* **2000**, *342*, 1946.
[4] M. A. Nuñez-Sánchez, A. González-Sarrías, R. García-Villalba, T. Monedero-Saiz, N. V. García-Talavera, M. B. Gómez-Sánchez, C. Sánchez-Álvarez, A. M. García-Albert, F. J. Rodríguez-Gil, M. Ruiz-Marín, F. A. Pastor-Quirante, F. Martínez-Díaz, F. A. Tomás-Barberán, J. C. Espín, M. T. García-Conesa, *J. Nutr. Biochem.* **2017**, *42*, 126.
[5] Y.-J. Surh, *Nat. Rev. Cancer* **2003**, *3*, 768.
[6] L. Ricciardiello, D. J. Ahnen, P. M. Lynch, *Nat. Rev. Gastroenterol. Hepatol.* **2016**, *13*. Available from: https://doi.org/10.1038/nrgastro.2016.56
[7] M. Larrosa, A. González-Sarrías, M. J. Yáñez-Gascón, M. V. Selma, M. Azorín-Ortuño, S. Toti, F. Tomás-Barberán, P. Dolara, J. C. Espín, *J. Nutr. Biochem.* **2010**, *21*, 717.
[8] S. G. Kasimsetty, D. Blalonska, M. K. Reddy, G. Ma, S. I. Khan, D. Ferreira, *J. Agric. Food Chem.* **2010**, *58*, 2180.
[9] S. K. Jaganathan, M. V. Vellayappan, G. Narasimhan, E. Supriyanto, *World J. Gastroenterol.* **2014**, *20*, 4618.
[10] R. Tzulker, I. Glazer, I. Bar-Ilan, D. Holland, M. Aviram, R. Amir, *J. Agric. Food Chem.* **2007**, *55*, 9559.
[11] N. Seeram, R. Lee, M. Hardy, D. Heber, *Sep. Purif. Technol.* **2005**, *41*, 49.
[12] D. Heber, *Herbal Medicine: Biomolecular and Clinical Aspect*, 2nd ed., CRC Press, Boca Raton, FL, USA **2011**, Ch. 10.
[13] O. Orgil, E. Schwartz, L. Baruch, I. Matityahu, J. Mahajna, R. Amir, *LWT Food Sci. Technol.* **2014**, *58*, 571.
[14] J. Li, F. Zhang, S. Wang, *Tumour Biol.* **2014**, *35*, 7475.
[15] M. M. Joseph, S. R. Aravind, S. Varghese, S. Mini, T. T. Sreelekha, *Mol. Med. Rep.* **2012**, *5*, 489.
[16] J. Boateng, M. Verghese, L. Shackelford, L. T. Walker, J. Khatiwada, S. Ogutu, D. S. Williams, J. Jones, M. Guyton, D. Asiamah, F. Henderson, L. Grant, M. DeBruce, A. Johnson, S. Washington, C. B. Chawan, *Food Chem. Toxicol.* **2007**, *45*, 725.
[17] N. Banerjee, H. Kim, S. Talcott, S. Mertens-Talcott, *Carcinogenesis* **2013**, *34*, 2814.
[18] M. I. Waly, A. Ali, N. Guizani, A. S. Al-Rawahi, S. A. Farooq, M. S. Rahman, *Asian Pac. J. Cancer Prev.* **2012**, *13*, 4051.
[19] M. I. Waly, A. S. Al-Rawahi, M. Al Riyami, M. A. Al-Kindi, H. K. Al-Issaei, S. A. Farooq, A. Al-Alawi, M. S. Rahman, *BMC Complement. Altern. Med.* **2014**, *14*, 60.
[20] H. Kohno, R. Suzuki, Y. Yasui, M. Hosokawa, K. Miyashita, T. Tanaka, *Cancer Sci.* **2004**, *95*, 481.
[21] N. A. H. Sadik, O. G. Shaker, *Dig. Dis. Sci.* **2013**, *58*, 2507.
[22] J. M. Amos-Landgraf, L. N. Kwong, C. M. Kendziorski, M. Reichelderfer, J. Torrealba, J. Weichert, J. D. Haag, K.-S. Chen, J. L. Waller, M. N. Gould, W. F. Dove, *PNAS* **2007**, *104*, 4036.
[23] G. Caderni, A. P. Femia, A. Giannini, A. Favuzza, C. Luceri, M. Salvadori, P. Dolara, *Cancer Res.* **2003**, *63*, 2388.
[24] A. P. Femia, A. Giannini, M. Fazi, E. Tarquini, M. Salvadori, L. Roncucci, F. Tonelli, P. Dolara, G. Caderni, *Cancer Prev. Res.* **2008**, *1*, 562.
[25] B. Cerdá, P. Periago, J. C. Espín, F. A. Tomás-Barberán, *J. Agric. Food Chem.* **2005**, *53*, 5571.
[26] J. C. Espín, R. González-Barrio, B. Cerdá, C. López-Bote, A. I. Rey, F. A. Tomás-Barberán, *J. Agric. Food Chem.* **2007**, *55*, 10476.
[27] A. de Conti, V. Tryndyak, I. Koturbash, R. Heidor, J. Kuroiwa-Trzmielina, T. P. Ong, F. A. Beland, F. S. Moreno, I. P. Pogribny, *Carcinogenesis* **2013**, *34*, 1900.
[28] S. J. Bultman, *Clin. Cancer Res.* **2014**, *20*, 799.
[29] A. P. Femia, C. Luceri, P. V. Soares, M. Lodovici, G. Caderni, *Int. J. Cancer* **2015**, *136*, E488.

[30] A. P. Femia, F. Salvianti, C. Luceri, P. Dolara, M. Salvadori, P. Pinzani, G. Caderni, *Int. J. Cancer* **2012**, *131*, 529.
[31] A. P. Femia, P. Dolara, C. Luceri, M. Salvadori, G. Caderni, *Int. J. Cancer* **2009**, *125*, 541.
[32] V. Pitozzi, A. Mocali, A. Laurenzana, E. Giannoni, I. Cifola, C. Battaglia, P. Chiarugi, P. Dolara, L. Giovannelli, *J. Gerontol. A Biol. Sci. Med. Sci.* **2013**, *68*, 371.
[33] C. Castagnoli, D. Alotto, I. Cambieri, R. Casimiri, M. Aluffi, M. Stella, S. T. Alasia, G. Magliacani, *Burns* **2003**, *29*, 759.
[34] M. A. Nunez-Sanchez, A. Gonzalez-Sarrias, M. Romo-Vaquero, R. Garcia-Villalba, M. V. Selma, F. A. Tomas-Barberan, M. T. Garcia-Conesa, J. C. Espìn, *Mol. Nutr. Food Res.* **2015**, *59*, 1274.
[35] U. A. Fischer, R. Carle, D. R. Kammerer, *Food Chem.* **2011**, *127*, 807.
[36] K. M. Tuohy, L. Conterno, M. Gasperotti, R. Viola, *J. Agric. Food Chem.* **2012**, 8776.
[37] M. Khatib, C. Giuliani, F. Rossi, A. Adessi, A. Al-Tamimi, G. Mazzola, D. Di Gioia, M. Innocenti, N. Mulinacci, *Food Chem.* **2017**, *235*, 58.
[38] A. P. Femia, C. Luceri, P. Dolara, A. Giannini, A. Biggeri, M. Salvadori, Y. Clune, K. J. Collins, M. Paglierani, G. Caderni, *Carcinogenesis* **2002**, *23*, 1953.
[39] A. P. Femia, P. Dolara, G. Caderni, *Carcinogenesis* **2004**, *25*, 277.
[40] D. E. Corpet, S. Taché, *Nutr. Cancer* **2002**, *43*, 1.
[41] D. E. Corpet, F. Pierre, *Cancer Epidemiol. Biomarkers Prev.* **2003**, *12*, 391.
[42] A. P. Femia, C. Luceri, F. Bianchini, M. Salvadori, F. Salvianti, P. Pinzani, P. Dolara, L. Calorini, G. Caderni, *Mol. Nutr. Food Res.* **2012**, *56*, 1353.
[43] L. Gamet, D. Daviaud, C. Denis-Pouxviel, C. Remesy, J. C. Murat, *Int. J. Cancer* **1992**, *52*, 286.
[44] M. Zhang, Q. Zhou, R. G. Dorfman, X. Huang, T. Fan, H. Zhang, J. Zhang, C. Yu, *BMC Gastroenterol.* **2016**, *16*, 84.
[45] J. A. Giménez-Bastida, A. González-Sarrías, M. Larrosa, F. Tomás-Barberán, J. C. Espín, M. T. García-Conesa, *Mol. Nutr. Food Res.* **2012**, *56*, 784.
[46] K. Jaskiewicz, E. Lancaster, L. Banach, A. Karmolinski, *Anticancer Res.* **1998**, *18*, 4641.