Flavanols from Japanese quince (Chaenomeles japonica) fruit suppress expression of cyclooxygenase-2, metalloproteinase-9, and nuclear factor-kappaB in human colon cancer cells

Katarzyna Owczarek1, Elżbieta Hrabec2, Jakub Fichna1, Dorota Sosnowska1, Maria Koziolkiewicz1, Jacek Szymański1 and Urszula Lewandowska1,2

1Department of Biochemistry, Faculty of Medicine, Medical University of Lodz, Łódź, Poland; 2Department of Medical Enzymology, Faculty of Medicine, Medical University of Lodz, Łódź, Poland; 3Institute of Technical Biochemistry, Faculty of Biotechnology and Food Sciences, Lodz University of Technology, Łódź, Poland; 4Central Scientific Laboratory, Faculty of Health Sciences, Medical University of Lodz, Łódź, Poland

Natural polyphenols and polyphenol-rich extracts have been found to possess preventive and therapeutic potential against several types of cancers, including colorectal cancer (CRC), which is an example of an inflammation-associated cancer. This study examines the chemopreventive effect of a Japanese quince (Chaenomeles japonica) fruit flavanol preparation (JQFFP) on colon cancer SW-480 cells. JQFFP, rich in procyanidin monomers and oligomers, was found to inhibit the SW-480 cell viability by 40% at 150 µM catechin equivalents (CE) after 72 h when compared to control, but it was non-toxic to normal colon fibroblast CCD-18Co cells. Furthermore, 100 µM CE JQFFP suppressed COX-2 mRNA expression to 36.7% of control values and protein expression to 77%. In addition, JQFFP reduced the MMP-9 protein expression (to 24% vs. control at 100 µM CE) and caused inhibition of its enzymatic activity (to 35% vs. control at 100 µM CE). Not only did JQFFP inhibit the COX-2 and MMP-9 levels, but it also reduced the NF-κB protein expression (to 65% of control) and phosphorylation of its p65 subunit (to 51%) at 100 µM CE. These results provide the first evidence that JQFFP inhibits COX-2, MMP-9, and NF-κB expression, suggesting that it has cytotoxic, anti-inflammatory, and anti-metastatic activities towards the colon cancer SW-480 cells.

Key words: flavanols, Japanese quince (Chaenomeles japonica), colon cells, COX-2, MMP-9, NF-κB

Received: 26 April, 2017; revised: 05 July, 2017; accepted: 10 July, 2017

INTRODUCTION

Plants constitute an important source of biologically-active compounds in foods which promote health and proper functioning of the organism, such as polyphenols, phytosterols, carotenoids, phospholipids, saturated and unsaturated fatty acids (Afrin et al., 2016). Research carried out in recent years indicates that natural polyphenols and polyphenol-rich extracts isolated from edible and therapeutic plants exhibit anti-inflammatory actions towards various cells, including human colon cancer cells. By modulating some signal transduction pathways, they can act as anti-inflammatory agents (Qian et al., 2015). Phenolic compounds could modulate the activities of some enzymes and other functional proteins which are involved in the inflammatory process (Shanmugam et al., 2011; Mitjavila & Moreno, 2012). Among molecular targets of anti-inflammatory agents are cyclooxygenase-2 (COX-2) and nuclear factor-kappaB (NF-κB) (Shanmugam et al., 2011; Tak & Firestein, 2001).

COX-2 catalyzes conversion of arachidonic acid to pro-inflammatory mediators, such as prostaglandins (in particular prostaglandin E2), which act as critical secondmessengers for immune processes, and stimulate various malignancies, such as colorectal carcinogenesis (Wang & Dubois, 2010; Dixon et al., 2013). Up-regulation of COX-2, and, consequently, prostaglandin E2, may be involved in the invasion and metastasis of colorectal cancer cells by stimulating expression of the matrix metalloproteinases (MMPs), which play a key role in the degradation of the extracellular matrix (ECM) (Kessenbrock et al., 2010). Moreover, aberrant or increased expression of COX-2 has been widely implicated in the pathogenesis of many cancer types including colorectal cancer (CRC) (Park et al., 2014). Increased expression of COX-2 has been found in both, premalignant and malignant colorectal tumors, than in the normal intestinal mucosa, and high levels of COX-2 have been associated with decreased cancer patient survival (Wang et al., 2010). Transcriptional upregulation of the COX-2 gene expression can be induced by various signaling pathways (Temraz et al., 2013). It has been reported that the promoter region of the COX-2 gene contains a number of sequences that specifically bind several transcriptional factors, including nuclear factor-κB (NF-κB) (Tazawa et al., 1994).

NF-κB, a transcription factor of the Rel/NF-κB family, comprised of a p50/p65 heterodimer, may control expression of genes whose proteins regulate many important cellular processes, such as inflammation, immune reactions, apoptosis, metastasis, angiogenesis, cell growth and proliferation (Lawrence, 2009). Additionally, it has been shown that NF-κB is constitutively activated in CRC and correlates with tumor progression (Kojima et al., 2004). Therefore, over-expression of COX-2, which can enhance malignant potential in CRC, may result from NF-κB activation.

In our previous study, a Japanese quince fruit flavanol preparation (JQFFP) demonstrated preventive and anti-cancer effects on several cell lines. JQFFP, particularly its fractions enriched in higher oligomers of procyanidins, inhibited activities of the type IV collagenases (MMP-2, MMP-9) secreted to the culture medium by human leukocytes.
kemia HL-60 cells and induced apoptosis in colon cancer Caco-2 cells (Stręc et al., 2007; Gozdach et al., 2011). Other studies had found that JQFP induces apoptosis, suppresses invasiveness and down-regulates many genes crucial for development and progression of breast and prostate cancer cells (Lewandowska et al., 2013a), and that an ethanolic extract of Chaenomeles speciosa Nakai induces apoptosis in a murine H22 cell line and inhibits tumor growth in the Kunming mice (Yao et al., 2013). A recent study by Strugala et al. had found that Chaenomeles speciosa Nakai (Japanese quince, JQ) extract inhibits activity of the cyclooxygenase enzymes (COX-1 and COX-2) in a cell-free in vitro assay (Strugala et al., 2016).

Following up on these findings, together with the fact that COX-2 is one of the pro-inflammatory mediators whose expression may be induced at the very early steps of colorectal carcinogenesis, the study presented here examines influence of JQFP on the COX-2 expression in the colon cancer SW-480 cells. It also assesses the influence of the tested preparation on MMP-9 and NF-κB activation in those cells.

MATERIALS AND METHODS

Chemicals. Acetonitrile, (+)-catechin, (-)-epicatechin, and vanillin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Quercetin glucoside and quercetin galactoside were obtained from Extrastynthese (Lyon, France). Chlorogenic acid and procyanidin B2 were purchased from PhytoLab GmbH & Co.KG (Vestenbergsgreuth, Germany). All other chemicals were reagent grade products purchased from POCH S.A. (Gliwice, Poland). Cell culture media and media supplements were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant materials. Ripe fruit of Japanese quince (Chaenomeles japonica L., Rosaceae) was collected from bushes planted in a yard in the central region of Poland (The Research Institute of Horticulture in Skieniewice) and stored at −20°C before polyphenol extraction.

Preparation of flavanol-rich extract from Japanese quince. Japanese quince dry flavanol extract was obtained according to Oszmiański (Oszmiański, 1992). In short, fresh pulp obtained from the Japanese quince fruit (0.8 kg) was extracted twice with 90% acetone (1:2.5 w/v), and then the pooled extracts were treated twice with trichloromethane (1:1 v/v) to remove the lipids. Phenolics, mainly procyanidins (flavanols), were extracted from the water phase with ethyl acetate, and the obtained solutions were concentrated under vacuum (Rotavapor RII, BUCHI). The final extract was precipitated from trichloromethane as (+)-catechin equivalents, total flavanol content, expressed as (+)-catechin equivalents, and total proanthocyanidin content, expressed as cyanidin.

Total phenolic content. The total phenolic content of the JQFP was determined by using the Folin-Ciocalteu reagent. Accurately weighed 20 mg of extract was dissolved in 10 mL of 10% aqueous dimethyl sulfoxide; then, 0.1–0.2 mL were mixed with 25 mL of water, 0.5 mL of Folin-Ciocalteu reagent and 5 mL of 20% sodium carbonate and made up to 50 mL with distilled water. The mixture was kept for 20 min at room temperature, after which the absorbance was read at 760 nm. (+)-Catechin was used as a reference standard, and the results were expresses as milligram (+)-catechin equivalents in 1 g of dry mass of extract.

Total flavanol content. The vanillin assay was performed as described previously (Lewandowska et al., 2013b). In short, a volume of 2 mL of a known dilution of the preparation solution was placed in two test tubes, and 4 mL of 1% (w/v) vanillin in 70% sulfuric acid (A) or 4 mL of 70% sulfuric acid (B) were added to the sample. Additionally, a blank was prepared by mixing 2 mL of water with vanillin solution (C). All test tubes were shaken in a bath of cold water to prevent the temperature from rising above 35 °C. After incubation in cold water for 15 min, the absorbance of samples A, B and C was read at 500 nm against a mixture of 2 mL of water and 70% sulfuric acid. The final absorbance is equal to the difference (A-B-C). The flavanols' content was calculated from a calibration curve, using (+)-catechin as a standard. Results were expressed as milligram (+)-catechin equivalents in 1 g of dry mass of extract.

Total proanthocyanidin content. The proanthocyanidins were determined after acid depolymerization to the corresponding anthocyanidins as described by Rösch (Rösch et al., 2003). 0.5–1.0 mg samples of dry flavanol extract were mixed with 10 mL of a solution of concentrated hydrochloric acid in n-butanol (1:9, v/v) and heated for 90 min in a boiling water bath. After cooling on ice, the absorbance of samples was measured at 550 nm. The content of proanthocyanidins was calculated with the molar extinction coefficient of cyanidin (ε=17360 L/mol cm and molar mass 287 g/mol) and was expressed as milligram cyanidin equivalents in 1 g of dry mass of extract.

Phenolics' identification by UPLC-Q-TOF-MS. Identification of polyphenol compounds was performed as described previously (Salaga et al., 2014) using the Acquity Ultra-performance Liquid Chromatography (UPLC) system coupled with a quadrupole-time of flight (Q-TOF) MS instrument (UPLC/Synapt Q-TOF MS, Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) source. Separation was achieved on an AcquityTM BEH C18 column (100 mm x2.1 mm i.d., 1.7 µm; Waters). The mobile phase was a mixture of 4.5% formic acid (A) and acetonitrile (B). The gradient program was as follows: initial conditions 99% (A); 12 min, 75% (A); 12.5 min, 100% (B); 13.5 min, 99% (A). The flow rate was 0.45 mL/min. The major operating parameters for the Q-TOF MS were set as follows: capillary voltage, 2.0 kV; cone voltage, 45 V; cone gas flow, 11 L/h; collision energy, 50 eV; source temperature, 100°C; desolvation temperature, 250°C; collision gas, argon; desolvation gas, nitrogen; flow rate, 600 L/h; data acquisition range, m/z 100–1000 Da; ionization mode, negative. The data were collected by Mass-LynxTM V4.1 software.

HPLC analysis of phenolic compounds. HPLC analysis was performed by using an analytical reversed-phase HPLC system (Dionex, Sunnyvale, CA, USA) with an EWS-3000/1 autosampler and LPG-3400A pump coupled to a photodiode array detector (Ultimate 3000), controlled by Chromeleon v. 6.8 software, according to Kucharska (Kucharska, 2012). Separation was performed on an Atlantis T3 (250 mm x4.6 mm i.d., 5 µm; Waters, Dublin, Ireland). The eluent was 4.5% formic acid (A) and acetonitrile (B). A gradient solvent system was used: 0–1 min, 5% (B); 1–6 min, 10% (B); 6–26 min, 20% (B); and 26–33 min, 100% (B). The flow rate was 1 mL/min, and the injection volume was 20 µL. Detector was set at 280 nm for flavan-3-ol derivatives and hydroxylable
tannin derivatives, and 360 nm for flavonol derivatives. The amounts of flavan-3-ol derivatives were expressed as (+)-catechin equivalents, hydrolyzable tannin derivatives as gallic acid equivalents, and flavonols as quercetin glucoside equivalents in 1 g of dry mass of extract.

**Cells and culture conditions.** SW-480 human colon cancer cells and CCD-18Co human colon fibroblast cells were purchased from the American Type Culture Collection (ATCC; ref: CCL-228 and CFR-1459) (LG Standards, Poland). SW-480 cells were cultured in the Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 1% MEM non-essential amino acids. The cells were grown in a humidified atmosphere at 37°C without CO₂. CCD-18Co cells were cultured in the Eagle’s minimum essential medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 1% MEM non-essential amino acids. Cells were grown in a humidified atmosphere with 5% CO₂ at 37°C. All experiments were carried out between passages 4 and 17. The cells were seeded in such a quantity that the confluence at the end of the experiment did not exceed 80% in the control wells. In the work presented here, JQFFP was tested within the concentration range of 25 to 150 µM catechin equivalents (CE), which corresponds to 7.3–43.5 µg CE/mL or 15.6–93.5 µg of preparation/mL. For all biosays, JQFFP was dissolved in a 70% ethanol solution in deionized water and then diluted to the final concentration with a serum-free medium. The final concentration of ethanol was lower than 0.01% (v/v). Controls for the JQFFP treatment were incubated with ethanol.

The experimental protocol was approved by the Ethics Committee of the Medical University of Lodz (No. RNN/88/15/KE).

**Cell viability assay.** Cell viability was assessed after 48 and 72 hour incubation with or without JQFFP (0–150 µM CE) by crystal violet staining, as described elsewhere (Lewandowska et al., 2013a). Briefly, the cells were harvested, suspended in the growth medium mentioned above, and seeded on 96-well plates (SW-480 and CCD-18Co were seeded at the densities of 10×10³ and 2×10³ per well, respectively). After 24 h, the cells were washed twice with PBS and then suspended in a medium with 3% FBS. Next, the cells were treated with JQFFP for 48 and 72 h. After culture, the medium was removed and the cells were fixed in situ with 4% formaldehyde solution in PBS for 30 min at room temperature. Then, the cells were washed twice with PBS (pH 7.4) and stained for 5 min at room temperature with 0.5% crystal violet dissolved in 25% aqueous solution of methanol. Unbound dye was washed out with deionized water and the cells were allowed to air dry. The dye was solubilized in 33% aqueous solution of acetic acid, while shaking for 30 min at room temperature by using a titer plate shaker (Lab-Line Instruments, Inc., Melrose Park, Illinois, USA). Optical density (OD) was measured by a microplate reader (iMarkTM, BioRad Laboratories) at a wavelength of 595 nm. Each experimental condition was replicated three times, and the percentage of cell viability was calculated against untreated cells.

**RNA isolation, cDNA synthesis and quantitative real time PCR.** RNA isolation, cDNA synthesis and quantitative polymerase chain reaction (Q-PCR), experiments were performed according to the method of Lewandowska and coworkers (2013a). Briefly, cells were incubated in serum-free media, with or without JQFFP (0–150 µM CE), for 16 h. Total RNA was isolated using TRIzol® reagent (InvitrogenTM, Carlsbad, CA) following the manufacturer’s guidelines. The concentration and purity of isolated RNA were determined spectrophotometrically at 260 and 280 nm (Colibri microvolume spectrometer, Titertek Berthold, Germany). cDNA synthesis was performed with 10 µg of total RNA in a total volume of 40 µL with a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Rockford, USA). Next, cDNA samples were diluted with RNase-free water to a total volume of 160 µL. Volumes of 2 µl (corresponding to 0.125 µg of total RNA) were used for Q-PCR which was carried out using the Rotor-Gene ™ 3000 (Corbett Research) thermocycler. Detection of Q-PCR products was done in a total volume of 20 µl with Rotor-Gene SYBER Green PCR Kit (Qiagen Inc., Crawley, UK). Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2) (Hs_PTS212_SG detecting transcript of NM 000963) primers were from Qiagen. The Q-PCR amplification procedure was comprised of the following stages: initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s (melting) and 60°C for 10 s (annealing and extension). Gene expression levels were normalized using two reference genes: beta-2-microglobulin (B2M) (Hs_B2M1_SG detecting transcripts of NM 004048/XM 002525459/XM 006725182) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs_GAPDH12_SG detecting transcripts of NM 001256799/NM 002046/NM 001289745/NM 001289746) (Qiagen Inc., Valencia, CA). Relative expression was calculated according to the Roche’s method using the Universal Human Reference RNA (Stratagene, La Jolla, CA) as a reference (Pfaffl et al., 2002).

**Protein preparation and Western blot analysis.** Cells were harvested, suspended in growth medium, and seeded in 75 cm² bottles. After 24 h, the cells were washed twice with PBS and then suspended in a serum-free medium. Subsequently, the cells were treated with JQFFP within the concentration range of 25–100 µM CE and incubated for 48 h. Control samples were incubated without JQFFP. Cell lysates were prepared using a Mammalian Cell Lysis Kit (Sigma-Aldrich Co. LLC, St. Louis, MO, USA), supplemented with the protease inhibitor mix M (Serva Electrophoresis, Heidelberg, Germany). The protein content of the cell lysates was then determined by using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein cell lysates (50 µg) were subjected to SDS-PAGE in 12–15% polyacrylamide gel and transferred onto a 0.22–0.45 mm nitrocellulose membrane (BioRad, Hercules, CA, USA). The membranes were blocked with 3% (w/v) non-fat milk in Tris-Buffered Saline with Tween (TBST) and incubated with primary antibodies at the following concentrations: goat anti-COX-2 (sc-1745, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:100; rabbit anti-

**NF-κB p65 (sc-109, Santa Cruz Biotechnology), 1:200; mouse anti-MMP-9 (sc-21733, Santa Cruz Biotechnology), 1:200; rabbit anti-actin (A5060, Sigma-Aldrich Co. LLC, St. Louis, MO, USA), 1:1000. Afterwards, secondary antibodies (horseradish peroxidase-conjugated) were used at the following concentrations: donkey anti-goat IgG (sc-2020, Santa Cruz Biotechnology), 1:20000; anti-rabbit IgG (A0545, Sigma-Aldrich Co. LLC, St. Louis, MO, USA), 1:100000; and anti-mouse IgG (sc-2005, Santa Cruz Biotechnology), 1:5000. The membranes were washed, and detection was performed using the enhanced chemiluminescence blotting detection system (SuperSignal West Pico Chemiluminescent Substrate, ThermoFisher Scientific, Rockford, USA). The intensity of the bands was quantified by densitometric analysis us-
ing the GelDoc™ EQ system with Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Immunoblots were normalized to the β-actin content.

**Enzyme-linked immuno-sorbet assay (ELISA).** Cells were suspended in the growth medium mentioned above and seeded in bottles (4×10^5/25 cm² bottle) and incubated for 24 h to allow attachment. The attached cells were washed twice with PBS and suspended in 5 mL of a serum-free medium, with or without JQFFP (0–100 µM CE), for 48 h. After 48 h, the medium was removed and the cells were washed with PBS, followed by incubation in the CytoBuster Protein Extraction Reagent (Novagen, Hercules, CA, USA) at room temperature for 5 min. Next, the lysates were cleared by centrifugation at 16,000×g for 5 min at 4°C, and the supernatants (cell extracts) were stored at −70°C for further analysis. The protein content of the cell lysates was then determined using Bradford reagent (BioRad, Hercules, CA, USA). Enzyme linked immunosorbent assay (CBA053, Calbiochem® COX-2 ELISA Kit, Merek Millipore) was used for quantitative determination of the COX-2 levels in cell lysates according to the manufacturer’s protocol. In short, standard, control, and lysate samples were added to 96-well plates coated with a rabbit anti-human COX-2 polyclonal antibody (capture antibody). COX-2 was then detected with a monoclonal anti-COX-2 antibody and a goat anti-mouse IgG HRP-conjugate. Subsequently, a tetramethyl-benzidine substrate was added to each well, giving a blue-colored product in the presence of the HRP-conjugate. Finally, the absorbance was measured at the wavelengths of 450 nm by a microplate reader and the COX-2 content was calculated according to a standard curve.

**Gelatin zymography assay and quantitative analysis of MMP-2 and MMP-9 secretion.** SW-480 cells were harvested, suspended in the growth medium mentioned above and seeded at the density of 8×10^4 per well on 96-well plates. After 24 h, the cells were washed twice with PBS and then suspended in a serum-free medium with various concentrations of JQFFP, or without it, for 48 h. Gelatin zymography of the cell culture media samples was performed following the method of Lewandowska and coworkers (2014). In short, the samples were dissolved in an electrophoresis sample buffer containing sodium dodecyl sulfate and subjected to electrophoresis in a 10% polyacrylamide gel embedded with gelatin (1 mg/mL) in the absence of β-mercaptoethanol. After electrophoresis, type IV collagenases (MMP-2 and MMP-9) were renatured by incubation with 2% Triton X-100, and the enzymatic reaction was allowed to proceed at 37°C for 21 h. Thereafter, the gels were stained for 1.5 h with 0.0125% Amido Black in 7% acetic acid and 20% ethanol. Type IV collagenases were visualized without destaining as transparent bands against the dark blue background of Amido Black-stained slab gels. Densitometry analysis was carried out using GelDoc™ EQ system with Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Phosphorylation detection by flow cytometry.** SW-480 cells were seeded at a density of 1.2×10^5 per 60 mm well in 5 mL of a culture medium (L-15) containing 10% FBS. After 24 h of initial incubation, the cells were washed twice with PBS and suspended in 5 mL of a serum-free L-15 and with 100 µM CE JQFFP. Control samples were incubated without JQFFP. The incubation lasted for 48 h. Next, the cells were fixed with the BD Cytofix Fixation Buffer (BD Biosciences), permeabilized at a concentration of 1×10³/mL with the Perm Buffer III, and stained according to the manufacturer of the PE-CE594 Mouse Anti-Human NF-κB p65 (pS529) (BD Biosciences, Bedford, MA, USA). The stained samples were immediately analyzed with the use of a flow cytometer (Canto II, BD Biosciences). Data was processed using FACSDiva 6.1.3 software (Becton Dickinson). Four independent experiments were carried out.

**Statistical analysis.** Statistical analyses were performed using PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Data is presented as mean ± S.D. or SEM, as indicated in the figure legends. The number of independent experiments is given in the figure legends. The statistical significance of differences between means was determined by a one-way ANOVA followed by a post hoc multiple comparison Newman-Keuls test. P values of <0.05 were considered to be statistically significant.

**RESULTS**

**Characterization of JQFFP composition**

The composition of JQFFP was determined by both, spectrophotometric and UPLC-Q-TOF-MS chromatographic methods (Table 1 and Table 2). A representative chromatogram for the analysis of JQFFP components is depicted in Fig. 1. Japanese quince fruit was revealed to be a rich source of highly diversified polyphenols: colorimetric analysis had found its total polyphenol content to be 465.31±32.22 mg/g, and flavanols constituted 47.16% (219.46±11.67 mg/g) of the preparation. The UPLC-Q-TOF-MS analysis showed that the predominant compounds in JQFFP were flavan-3-ol derivatives such as procyanidin monomers and oligomers, ranging from dimers to pentamers, which accounted for about 92.58%, with chlorogenic acid constituting 6.34%.

**Effect of JQFFP on viability of SW-480 and CCD-18Co cells**

As demonstrated in Fig. 2, the influence of JQFFP on viability of SW-480 colon cancer cells (Fig. 2A) and CCD-18Co colon fibroblast cells (Fig. 2B) was also assessed. The cells were treated with various concentrations of JQFFP ranging from 25 µM CE to 150 µM CE. JQFFP reduced SW-480 cell viability after 48 and 72 h of incubation. After the 72 h incubation with JQFFP, the number of live SW-480 cells decreased in a concentration-dependent manner: falling by 20% with regard to control (untreated cells) at 75 µM CE, 25% at 100 µM CE, 28% at 125 µM CE and 40% at 150 µM CE. In addition, statistically significant viability inhibition was observed. After the shorter incubation time (48 h) with JQFFP, significant inhibition of cell viability was detected in the concentration range of 125–150 µM CE (15% and 30%, respectively). It is worth noting that JQFFP did not influence the CCD-18Co cell viability after either

<table>
<thead>
<tr>
<th>Table 1. General characteristic of JQFFP (mg/g dry preparation; mean ± S.D., n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total polyphenols</strong></td>
</tr>
<tr>
<td><strong>Total flavanols</strong></td>
</tr>
<tr>
<td><strong>Total proanthocyanidins</strong></td>
</tr>
</tbody>
</table>

* Determined by Folin-Ciocalteu reagent as (+)-catechin equivalents.

* Determined by vanillin-catechin reagent as (+)-catechin equivalents.
Table 2. UPLC-ESI/MS data for polyphenols identified in JQFFP (mg/g dry preparation)

<table>
<thead>
<tr>
<th>Peak number</th>
<th>$R_t$ (min)</th>
<th>[MS-H] (m/z)</th>
<th>MS/MS (m/z)</th>
<th>Compound</th>
<th>Chemical entity</th>
<th>Quantity (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.61</td>
<td>577,1349</td>
<td>407,0768/289,0688/125,0241</td>
<td>procyanidin dimer</td>
<td>flavan-3-ols</td>
<td>21.07</td>
</tr>
<tr>
<td>2</td>
<td>2.72</td>
<td>577,1349</td>
<td>407,0768/289,0688/125,0242</td>
<td>procyanidin dimer</td>
<td>flavan-3-ols</td>
<td>21.07</td>
</tr>
<tr>
<td>3</td>
<td>2.91</td>
<td>865,1905</td>
<td>577,1349/407,0768/289,0727/125,0218</td>
<td>procyanidin trimer</td>
<td>flavan-3-ols</td>
<td>0.45</td>
</tr>
<tr>
<td>4</td>
<td>3.07</td>
<td>865,1906</td>
<td>577,1349/407,0768/289,0768/125,0264</td>
<td>procyanidin trimer</td>
<td>flavan-3-ols</td>
<td>0.45</td>
</tr>
<tr>
<td>5</td>
<td>3.22</td>
<td>289,0723</td>
<td>151,0394/125,0264</td>
<td>(+)-catechin</td>
<td>flavan-3-ols</td>
<td>0.40</td>
</tr>
<tr>
<td>6</td>
<td>3.62</td>
<td>353,0879</td>
<td>191,0553</td>
<td>chlorogenic acid</td>
<td>phenolic acid</td>
<td>17.68</td>
</tr>
<tr>
<td>7</td>
<td>3.81</td>
<td>1153,2637</td>
<td>573,1207/287,0610</td>
<td>procyanidin tetramer</td>
<td>flavan-3-ols</td>
<td>3.08</td>
</tr>
<tr>
<td>8</td>
<td>3.98</td>
<td>577,1349</td>
<td>407,0810/289,0723/125,0241</td>
<td>procyanidin B2</td>
<td>flavan-3-ols</td>
<td>72.08</td>
</tr>
<tr>
<td>9</td>
<td>4.77</td>
<td>289,0723</td>
<td>151,0394/125,0264</td>
<td>(-)-epicatechin</td>
<td>flavan-3-ols</td>
<td>43.30</td>
</tr>
<tr>
<td>10</td>
<td>5.36</td>
<td>1153,2568</td>
<td>865,1906/577,1349/289,0688</td>
<td>procyanidin tetramer</td>
<td>flavan-3-ols</td>
<td>33.63</td>
</tr>
<tr>
<td>11</td>
<td>5.42</td>
<td>1441,3274</td>
<td>1153,2706/863,1810/575,1109/289,0688</td>
<td>procyanidin pentamer</td>
<td>flavan-3-ols</td>
<td>24.77</td>
</tr>
<tr>
<td>12</td>
<td>5.53</td>
<td>431,1887</td>
<td>287,0540</td>
<td>procyanidin</td>
<td>flavan-3-ols</td>
<td>11.10</td>
</tr>
<tr>
<td>13</td>
<td>7.18</td>
<td>463,0887</td>
<td>301,0319/271,0245</td>
<td>quercetin 3-O-galactoside</td>
<td>flavonols</td>
<td>1.27</td>
</tr>
<tr>
<td>14</td>
<td>7.29</td>
<td>577,1349</td>
<td>407,0727/289,0688/125,0241</td>
<td>procyanidin dimer</td>
<td>flavan-3-ols</td>
<td>4.01</td>
</tr>
<tr>
<td>15</td>
<td>7.37</td>
<td>865,1967</td>
<td>577,1398/407,0768/289,0768/125,0264</td>
<td>procyanidin trimer</td>
<td>flavan-3-ols</td>
<td>4.01</td>
</tr>
<tr>
<td>16</td>
<td>7.44</td>
<td>463,0887</td>
<td>301,0319/271,0245</td>
<td>quercetin 3-O-glucoside</td>
<td>flavonols</td>
<td>1.71</td>
</tr>
</tbody>
</table>

Flavan-3-ol derivatives were determined by HPLC at 280 nm as (–)-epicatechin

Figure 1. Base peak chromatogram of the JQFFP obtained by an MS data collection technique (UPLC-Q-TOF/MS ES) in a negative ionization mode.

The lower chromatogram presents the sample before fragmentation, and the upper chromatogram after fragmentation. Peaks are labeled according to Table 2.
48 or 72 h incubation throughout the whole concentration range chosen for the study (Fig. 2B). The obtained results indicate that the tested JQFFP has an inhibitory effect on colon cancer cell viability.

JQFFP inhibits both, COX-2 mRNA and protein expression, in SW-480 cells

mRNA expression was measured by Q-PCR. As presented in Fig. 3A, the expression of COX-2 mRNA was significantly reduced in a concentration-dependent manner following 16 h incubation with JQFFP. At concentrations of 25, 50, and 100 µM CE, the COX-2 expression was reduced to 66.9%, 76.2%, and 36.7%, respectively, when compared to control. Subsequently, it was determined whether the differences in COX-2 protein expression resulted from an altered COX-2 mRNA expression in SW-480. Western blot analysis confirmed the ability of JQFFP to inhibit COX-2 expression not only at the level of mRNA, but also at the protein level, as shown in Fig. 3B. The observed effect was dependent on the concentration of JQFFP, reaching 91%, 86%, and 77% for 25, 50, and 100 µM CE, respectively, after 48 h of incubation. Also, a similar tendency was observed in another study of quantitative determination of COX-2 protein in cell lysates by ELISA. As shown in Fig. 3C, JQFFP down-regulated the expression of COX-2 protein in SW-480 cells. At JQFFP concentrations of 25, 50, and 100 µM CE, the expression level of pro-inflammatory COX-2 protein decreased to 93%, 78%, and 72%, respectively, versus untreated control. The above-mentioned results indicated that JQFFP can reduce the COX-2 levels (mRNA and protein) in the SW-480 cell
line and in consequence exerts an anti-inflammatory effect in those cells.

**JQFFP inhibits MMP-9 protein expression**

JQFFP treatment caused a statistically significant and concentration-dependent reduction in protein expression of MMP-9, as determined by Western blotting methods. JQFFP treatment caused a statistically significant and concentration-dependent reduction in protein expression of MMP-9, as determined by Western blotting methods. After 48 h of JQFFP treatment, protein expression of MMP-9 decreased to 61%, 54%, and 24%, respectively, vs. control. After 48 h of JQFFP treatment, protein expression of MMP-9 decreased to 61%, 54%, and 24%, respectively, vs. control.
incubation with JQFFP, the activity of MMP-9 secreted to the culture medium by SW-480 cells was considerably reduced and, as shown in Figure 4B, at the concentrations of 25, 50, 75, and 100 µM CE, the inhibition reached 12%, 19%, 23%, and 35%, respectively, which clearly demonstrates a concentration-dependent effect. Additionally, gelatin zymography had found the tested preparation to inhibit secretion of the MMP-2 (Fig. 4C). This inhibition of MMP-9 by JQFFP suggests that it acts as an antimitastatic and antiangiogenic agent.

**JQFFP inhibits both, the NF-κB protein expression and NF-κB p65 (Ser 529) phosphorylation, in SW-480 cells**

Due to the fact that activities of COX-2 and MMP-9 are highly dependent on the transcription factor NF-κB, the next stage of our research was set to assess the impact of JQFFP on the NF-κB protein expression. Western blot studies revealed that JQFFP caused a statistically significant and concentration-dependent reduction in the expression of the NF-κB transcription protein factor (Fig. 5A). At JQFFP concentrations of 25, 50, and 100 µM CE, expression of the NF-κB protein decreased to 88%, 71%, and 65%, respectively, versus control. As NF-κB proteins, such as p65, require phosphorylation within their transactivation domain to optimally regulate expression of multiple target genes, including COX-2 and MMP-9, the next stage assessed impact of JQFFP on phosphorylation of the p65 subunit. The phosphorylation level of p65 was determined using a monoclonal antibody recognizing the phosphorylated serine 529 (pS529) in the transactivation domain of the NF-κB p65 subunit. Flow cytometry analysis revealed that at a JQFFP concentration of 100 µM CE, the phosphorylation level of the p65 subunit was reduced by 49% in respect to control (Fig. 5B and 5C). Our findings indicate that JQFFP has the ability to decrease both, the expression of NF-κB protein and the phosphorylation of the NF-κB p65 subunit, in the SW-480 cells.

**DISCUSSION**

A growing body of evidence suggests that dietary plant-derived compounds, including polyphenols, have anti-inflammatory and anticancer properties that can be mediated through COX-2 (Shanmugam et al., 2011). Additionally, evidence suggests existence of an association between CRC and COX-2 over-expression by creating a microenvironment conducive for the development of pre-neoplastic lesions (Temraz et al., 2013). Thus, selective inhibition of the COX-2 enzyme could hinder the development and progression of CRC. Research of celecoxib, a cyclooxygenase-2 selective inhibitor, has demonstrated that it can effectively decrease the number of colorectal polyps (Steinbach et al., 2000). Likewise, in clinical trials carried out in 1561 patients, celecoxib significantly reduced the risk of colorectal adenomas within three years after polypectomy. Unfortunately, it was associated with serious adverse events related to a cardiovascular disease (Arber et al., 2006, ClinicalTrials.gov number, NCT00005094). Due to the fact that celecoxib and other non-steroidal anti-inflammatory drugs (NSAIDs) can cause severe side effects (e.g., gastrointestinal ulceration, bleeding, and platelet dysfunction), an increasing number of studies has focused on finding new, low or non-toxic anti-inflammatory drugs. Compounds from natural sources might be a promising alternative strategy to synthetic COX-2 inhibitors for inflammation prevention and therapy. Numerous studies in different cell lines and animal models suggest that polyphenols and polyphenol-rich extracts have a preventive, anti-inflammatory effect against CRC (Sałaga et al., 2014; Zicińska et al., 2015).

Our previous study had found that a JQFFP exhibits a potent antiproliferative effect against breast and prostate cancer cells, inhibits their invasiveness, and decreases expression of several genes involved in apoptosis, angiogenesis, and metastasis (Lewandowska et al., 2013a). The work presented here, investigates the potential of JQFFP as an anti-inflammatory agent in colon cancer cells. The initial step was to determine the impact of JQFFP, found to be rich in procyanidins monomers and oligomers (Table 2) on the viability of human colon cancer cells (SW-480) and normal human colon fibroblasts (CCD-18Co). The tested flavanol preparation had demonstrated opposite effects towards colon cancer cells (SW-480) and normal colon fibroblast cells (CCD-18Co) (Fig. 2A and 2B); JQFFP reduced colon cancer cell viability in a time- and concentration-dependent manner but was not toxic to normal cells. Such results indicate that JQFFP exhibits an antiproliferative effect against SW-480 cells and may act as a protective agent towards CCD-18Co fibroblast cells which are part of the gut barrier and constitute an important component in the maintenance of the intestinal mucosa (Ivanov et al., 2010). These observations are similar to our previous report demonstrating that the JQFFP suppresses the growth of both, highly metastatic breast and prostate cancer cells (MDA-MB-231 and DU 145) but did not decrease the normal PNT1A human prostate cell number (Lewandowska et al., 2013a). Our results are in accordance with other literature data regarding the sensitivity of various types of cells to growth inhibitors of plant origin (Carvalho et al., 2010). For instance, Dias and coworkers (2014) found that a polyphenol extract from acai (Euterpe oleracea Mart.) does not influence the CCD-18Co cell viability at all, but significantly reduces growth of the SW-480 cell line. Likewise, previous studies have found that normal and cancer cells originated from the gastrointestinal tract (Caco-2, HT-29, IEC-6) have variable sensitivities to the antiproliferative activity of flavanols obtained from the Japanese quince fruit (Gorlach et al., 2011). SW-480 was chosen for the study as it is known to be inhibited by JQFFP.

Our findings indicate that JQFFP down-regulated COX-2 mRNA expression in SW-480 at each concentration of the tested flavanol preparation (63% vs. control at 100 µM CE) (Fig. 3A). Furthermore, we have shown that inhibition of COX-2 mRNA expression by JQFFP correlated with lower levels of COX-2 protein in a concentration-dependent manner, as evidenced by both, Western blot and ELISA (Fig. 3B and 3C). This down-regulation of COX-2 by JQFFP observed in the SW-480 cell line suggests that it possesses an anti-inflammatory activity. These findings are in agreement with those of a recent study which had shown that Japanese quince (IQ) extract has the capacity to inhibit the COX-1 and COX-2 enzymes. It should be noted, however, that this inhibition of COX-1 and COX-2 by IQ was analyzed in a cell-free test for COX-2 (Struggala et al., 2016). Besides this, the anti-inflammatory activity of JQFFP is consistent with the previously reported anti-inflammatory activity of a 10% ethanol fraction of Chaenomeles speciosa observed on carrageenan-induced rat paw edema (Li et al., 2009).

Furthermore, it has been found that COX-2 expression may be associated with the invasive and metastatic properties of CRC tumor cells (Chen et al., 2001). In one such study, Tsuji and coworkers (1997) had demonstrated that COX-2-overexpression in the Caco-
CONCLUSION

Our results clearly demonstrate that Japanese quince fruit flavanol preparation reduces the SW-480 colon cancer cell viability. Importantly, JQFFP did not cause any reduction in the number of normal CCD-18Co fibroblast cells, which are an essential component in the maintenance of the intestinal mucosa. Furthermore, this is the first report to identify the inhibition of both, the COX-2 and MMP-9 expression by JQFFP, which suggests that it has anti-inflammatory and anti-invasive activities towards colon cancer cells. These anti-inflammatory, anti-invasive, and anti-proliferative effects of JQFFP are linked to reduced NF-κB activation in the SW-480 cells, which is one of the significant mechanisms for the prevention of colorectal cancer. Our results indicate that JQFFP may be used as a natural source of anti-inflammatory and chemopreventive agents for CRC. Due to its natural origin, JQFFP is less likely to cause severe side effects than synthetic drugs, including NSAIDs.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

We gratefully acknowledge Alicia Kucharska PhD, DSc from the Wroclaw University of Environmental and Life Sciences for determining the composition of polyphenolic compounds by UPLC-QTOF-MS. This work was financed by the Medical University of Lodz (No. 503/1-156-04/503-01) and by the Polish Ministry of Science and Higher Education (Project No. NN 312-446-840).

REFERENCES

molecules21020169.

NEJMoa061652.

ja090386d.


2 cells increases activity of MMPs and stimulates their ability to promote endothelial cell motility through a matrix, which could be abrogated by treatment with COX inhibitors. It is also well known that overexpression of MMPs, particularly MMP-9, may be one part of the multi-step process that leads to neoplastic cell proliferation and metastasis in CRC (Cho et al., 2004), the next stage of our study had examined whether the COX-2 and MMP-9 activity inhibition by JQFFP in the SW-480 cells is mediated through NF-κB. We have found that JQFFP diminishes expression of the NF-κB protein in those cells (35% vs. control at 100 µM CE) (Fig. 5A). Furthermore, at the concentration of 100 µM CE, the tested JQFFP also reduced phosphorylation at serine 529 of the p65 subunit (49% vs. control) (Fig. 5B). It should be emphasized that JQFFP not only inhibited the NF-κB expression at the level of translation, but also phosphorylation of the p65 functional domain, resulting in the weakening of the transactivation potential of NF-κB in those cells. Our results are in line with reports on the suppression of COX-2, MMP-9, and NF-κB activation by other natural polyphenols and polyphenol-rich extracts in various experimental models in both, the CRC and other cancers (Afrin et al., 2016; Shanmugam et al., 2011; Rodríguez-Ramiro et al., 2013).


Kucharska AZ (2012) Active compounds of cornelian cherry fruit (Cornus mas L.), Publishing House of University of Wroclaw.


