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Flavanols from Evening Primrose (*Oenothera paradoxa*) Defatted Seeds Inhibit Prostate Cells Invasiveness and Cause Changes in *Bcl-2/Bax* mRNA Ratio

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ABSTRACT: In this study, we assessed the influence of an evening primrose flavanol preparation (EPFP) on proliferation and invasiveness of human prostate cancer cells (DU 145) and immortalized prostate epithelial cells (PNT1A). We report for the first time that EPFP reduces DU 145 cell proliferation (IC_{50} = 97 μM GAE for 72 h incubation) and invasiveness (by 24% versus control at 75 μM GAE). EPFP strongly inhibited PNT1A invasiveness in a concentration-dependent manner (by 67% versus control at 75 μM GAE) and did not cause a reduction in their proliferation. Furthermore, EPFP inhibited the activities of MMP-2 and MMP-9 secreted to culture medium by PNT1A cells by 84% and 34% versus control at 100 μM GAE, respectively. In the case of DU 145, MMP-9 activity at 100 μM GAE was reduced by 37% versus control. Moreover, the evening primrose seed flavanols suppressed the expression of selected genes (*MMP-1*, *MMP-9*, *MMP-14*, *c-Fos*, *c-Jun*, and *VEGF*) and also caused favorable changes in *Bcl-2/Bax* mRNA ratio which render DU 145 cells more sensitive to apoptosis-triggering agents. An additional confirmation of the proapoptotic activity of EPFP toward DU 145 was visualization of characteristic apoptotic bodies by DAPI staining. In conclusion, this study suggests that EPFP may increase apoptosis and reduce angiogenesis of prostate cancer cells.

KEYWORDS: *flavanols, evening primrose (Oenothera paradoxa), prostate cancer, DU 145, PNT1A, matrix metalloproteinases, invasiveness, apoptosis*

INTRODUCTION

Evening primrose (*Oenothera* sp.) is widely distributed in Central and South America as well as in Europe. Plants belonging to this genus are investigated mainly due to the fact that their seeds contain high amounts of unsaturated fatty acids, particularly linoleic acid, but also *cis*-6,9,12-oktadecatrienoic acid (γ-linolenic acid, GLA).[1] Defatted seeds of evening primrose are a waste product of pharmaceutical and cosmetic industries. As the seeds are rich in phenolics, which constitute approximately 10% of their dry mass, they are used in some countries (for instance, New Zealand) as a cheap source of antioxidants.[2,3] Health beneficial properties of polyphenol-rich extracts from evening primrose seeds result not only from the antioxidant activity of their constituents but also from other activities. There are reports on apoptosis induction by evening primrose constituents.[4−6] In 2010 two additional works on the bioactivities of the evening primrose seed phenolics were published. The first concerned the inhibition of reactive oxygen species generation and suppression of the release of leucotriene B4, IL-8, elastase, and myeloperoxidase by human neutrophils by an extract from evening primrose seeds and one of its constituents (pentagalloylglucose, PGG).[7] In the second work evening primrose phenolics were demonstrated to sensitize melanoma HTB-140 and liver cancer HepG2 cells to vincristine.[8] Recently, Gorlach et al.[9] reported a proapoptotic activity of an evening primrose seed extract toward human colon cancer cells (Caco-2).

Literature data indicate that matrix metalloproteinases (MMPs) are involved in all stages of carcinogenesis: initiation, angiogenesis, primary tumor growth, and metastasis.[10] Angiogenesis is one of the most important stages of cancer development, and MMPs play a key role in this process. Therefore, down-regulation of expression and/or activity of type IV collagenases (MMP-2 and MMP-9) is one of strategies of angiogenesis inhibition, which in turn results in suppression of tumor growth, cancer cell migration and invasion, and metastasis.[11]

Taking into account the essential role of MMPs in carcinogenesis, one can state that they are important molecular targets of anticancer therapy.[12] However, although the research in this field is ongoing, no synthetic MMP inhibitor has been successful in clinical trials owing to lack of efficiency and strong side effects, particularly within the muscular and skeletal systems.[11−13] For this reason there is a search for natural inhibitors of those enzymes, and polyphenols are among groups of compounds investigated in that respect.[11,14,15]

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males worldwide, accounting for estimated 14% (903,500) of the total new cancer cases and 6% (258 400) of the total cancer deaths

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in 2008.[16] The country with the highest prostate cancer incidence rate is the United States, and the respective values for males in U.S. in 2012 are 29% (241 740) of the total new cancer cases and 9% (28 170) of the total cancer deaths.[17] Therefore, in our studies we assessed anticancer (mainly antiangiogenic) activities of flavanols from defatted seeds of evening primrose (*Oenothera paradoxa*) toward a human prostate cancer cell line.

MATERIALS AND METHODS

Materials. An industrial evening primrose (*Oenothera paradoxa* Hudziok) seed cake left from cold oil processing was provided by pharmaceutical company Agropharm S.A./Adamed Group (Tuszyn, Poland). The seed cake was stored in a closed jar in a refrigerator. Gallic acid, ellagic acid, (+)-catechin, (−)-epicatechin, quercetin, and methyl gallate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dimer B1, dimer B2, and trimer C1 were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). HPLC grade acetonitryle was purchased from J. T. Baker (Griesheim, Germany). All other chemicals were reagent grade products purchased from POCH S.A. (Gliwice, Poland).

Flavanol Isolation and Characterization. Evening primrose flavanol preparation was obtained according to the procedure described in the Polish patent (PL 169082 B1).[18] Industrial defatted seed cake was milled and additionally defatted with hexane (1:5 w/v; three times for 15 min). Polyphenols were extracted from defatted seeds with 90% aqueous solution of acetone (1:2.5 w/v, twice, for 30 min) by stirring at room temperature. The obtained crude extract was treated twice with chloroform in the ratio of 1:1 (v/v). The aqueous phase was saturated with sodium chloride to remove flavanol polymers. Flavanol monomers and oligomers present in the solution were extracted three times with ethyl acetate in the ratio of 1:1 (v/v). After freezing of water at −18 °C for 24 h, the ethyl acetate phase was concentrated under vacuum at 40 °C. Then, flavanols were precipitated from ethyl acetate with the use of chloroform. The resulting preparation is referred to as evening primrose flavanol preparation (EPFP) in this work. The dry EPFP was stored at −20 °C prior to further analyses.

The preparation was characterized in terms of the following: total polyphenol content by Folin−Ciocalteu procedure and expressed as milligrams of gallic acid equivalents (GAE) per gram of dry preparation;[19] total flavanol content by vanilin procedure and expressed as milligrams of (+)-catechin equivalents per gram of dry preparation;[20] as well as total proanthocyanidin content after acid hydrolysis in butanol environment and expressed as milligrams of cyanidin equivalents per gram of dry preparation.[21] Ellagitannins and gallotanins (after acid hydrolysis in methanol environment, 20 h, 85 °C) were determined by analytical reversed-phase HPLC system with the use of a Eurospher-100 C18 column (250 mm × 4.6 mm, 5 μm) (Knauer, Berlin, Germany). Binary mobile phase and gradient program were the same as those used to determine the phenolic profile (described below). Ellagitannin content determined at 254 nm was expressed as ellagic acid and gallotannin content determined at 280 nm was expressed as methyl gallate.[22]

Phenolic profile was determined using analytical reversed-phase HPLC system (Waters) with an autosampler 2707 and a binary HPLC pump 1525 coupled to a 996 photodiode array detector (2998), controlled by Waters Breeze 2 software (Waters, Milford, MA). The column used was 250 mm × 4.6 mm (5 μm), SYMMETRY C18 (Waters). The binary mobile phase according to Dyrby et al.[23] consisted of water and formic acid in the ratio of 90:10 (v/v), respectively (solvent A); water, acetonitrile, and formic acid in the ratio of 49:50:10 (v/v/v), respectively (solvent B). The separation of phenolics was performed using the following gradient program with a flow rate of 1 mL/min: 0 min, 88%A + 12%B; 26 min, 70%A + 30%B; 40−43 min, 0%A + 100%B; 48−50 min, 88%A + 12%B. The flow rate was 1 mL/min, and the injection volume was 20 μL. UV−vis spectra were recorded in the range 200−500 nm. Detector was set at 280 nm for flavanols and hydroxybenzoic acid derivatives, at 320 nm for hydroxycinnamic acid derivatives, and at 360 nm for flavonols. Peak identification was carried out by comparison of retention time and diode array spectral characteristics with the standards.

Cells and Culture Conditions. Human prostate cancer cells DU 145 and PNT1A immortalized prostate epithelial cells were purchased from Health Protection Agency Culture Collections (London, U.K.). DU 145 cells were cultured in MegaCell Dulbecco's Modified Eagle's Medium (MC DMEM) supplemented with 3% fetal bovine serum (FBS), 4 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin, 1.25 μg/mL amphotericin B, 1 mM sodium pyruvate, 1% MEM nonessential amino acids. PNT1A cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 1.25 μg/mL amphotericin B. Cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were seeded in such quantity that the confluence at the end of the experiment did not exceed 80% in control wells. In the present work EPFP was tested within the concentration range from 25 to 100 μM gallic acid equivalents (GAE), which corresponds to 4.3−17.0 μg GAE/mL or 4.6−18.3 μg of preparation/mL. For all bioassays EPFP was dissolved in 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 EPFP treatment were incubated with ethanol. The culture media were not changed during the incubation with the tested preparation. (+)-Catechin (CAT), gallic acid (GA), and (−)-epigallocatechin 3-gallate (EGCG) were dissolved in serum-free medium.

Proliferation Assay. Cell proliferation was assessed after 24, 48, and 72 h incubation with or without EPFP (0−100 μM GAE) by crystal violet staining. Crystal violet staining is a colorimetric method which dyes cellular nuclei. The staining was performed according to Henriksson et al.[24] with slight modifications. Briefly, the cells were harvested, suspended in the growth medium mentioned above, and seeded on 96-well plates (PNT1A and DU 145 were seeded at the densities of 2.5×10^3 and 2.0×10^3 per well, respectively). After 24 h, the cells were washed twice with PBS and then suspended in medium without FBS. Next, the cells were exposed to EPFP, CAT, GA, and EGCG for 24, 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 with 0.5% crystal violet dissolved in 25% aqueous solution of methanol for 5 min at room temperature. 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. Optical density (OD) was measured by a microplate reader (iMark, BioRad Laboratories) at the wavelength of 595 nm.

Invasion Assay. Invasion studies were conducted using the Matrigel BM matrix assay developed for measurement of tumor cell invasiveness, as described previously.[25] We used BioCoat Matrigel invasion chambers (24-well cell culture inserts containing an 8.0-μm PET membrane with a uniform layer of Matrigel [Becton Dickinson, Bedford, MA]). The lower chamber contained medium with 10% FBS as a chemoattractant. Cells were resuspended in serum-free medium with or without EPFP (0−75 μM GAE), and plated onto the upper chamber (1×10^5 cells/500 μL) according to the manufacturer's recommendations. The chambers were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. After incubation the nonmigrated cells in the upper chamber were gently scraped away, and adherent cells present on the lower surface of the insert were stained with crystal violet, photographed, and measured using NIH ImageJ analysis software.

Gelatin Zymography. In order to determine the influence of EPFP on MMP-9 activity (92-kDa Type IV collagenase), the enzyme expression was stimulated with tumor necrosis factor α (TNF-α) and/or 12-*O*-tetradecanoylphorbol-13-acetate (TPA).[26] Concentrations of the stimulants were chosen experimentally for each cell line by zymographic analysis (data not shown). We chose the lowest concentrations at which MMP-9 stimulation was observed, namely, 5 ng/mL TPA for PNT1A, 10 ng/mL TNF-α, and 10 ng/mL TPA for DU 145. The cells were harvested, suspended in the growth medium

mentioned above, and seeded on 96-well plates (PNT1A were seeded at the density of 3.5×10^3 per well and DU 145 at the density of 5×10^3 per well). After 24 h, the cells were washed twice with PBS. Next, the cells were incubated in serum-free media in the presence of above-mentioned stimulants and with or without various concentrations of EPFP (0−100 μM GAE) and EGCG (0−100 μM) for 48 h. Gelatin zymography of cell culture media samples was performed as described previously.[27] Briefly, the same volumes of media (usually 20 μL) were dissolved in electrophoresis sample buffer containing sodium dodecyl sulfate (SDS) and subjected to electrophoresis in a 10% polyacrylamide gel embedded with gelatin (1.5 mg/mL) in the absence of β-mercaptoethanol. After electrophoresis type IV collagenases were renatured by incubation with 2.5% Triton X-100, and the enzyme 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 activities (MMP-2 and MMP-9) were visualized without destaining as transparent bands against the dark blue background of Amido Black-stained slab gels. The intensity of bands was quantified by densitometric analysis using GelDoc EQ system with Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA).

Quantitative Real Time RT-PCR (Q-PCR). PNT1A and DU 145 cell lines were harvested, suspended in the growth medium mentioned above, and seeded per 60.1 cm^2 culture dishes (PNT1A were seeded at the density of 7×10^5 per well and DU 145 at the density of 9×10^5 per well). After 24 h, the cells were washed twice with PBS. Next, the cells were incubated in serum-free media with or without various concentrations of EPFP (0−75 μM GAE), for 16 h. Total RNA was extracted using TRIzol reagent (InvitrogenTM, Carlsbad, CA) according to the manufacturer's guidelines. The concentration and purity of isolated RNA were determined spectrophotometrically at the wavelengths of 260 and 280 nm. Synthesis of cDNA was performed from 10 μg of total RNA in the total volume of 70 μL using ImProm-II reverse transcriptase (Promega, Madison, WI). Next, cDNA samples were diluted with sterile deionized water to the total volume of 140 μL. Volumes of 2 μL (corresponding to 0.14 μg of total RNA) were used for Q-PCR which was performed using the Rotor-Gene 3000 (Corbett Research) thermocycler. Detection of Q-PCR products was performed with SensiMixSYBER Low-ROX Kit (Bioline, London, U.K.). Gene expression levels were normalized using two reference genes: microglobulin (*B2M*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Relative expression was calculated according to the Roche's method using Universal Human Reference RNA (Stratagene, La Jolla, CA) as a reference.[28] Sequences of primers, annealing temperatures, and the lengths of products are presented in Table 1.

Detection of Apoptosis by 4′,6-Diamidino-2-Phenylindole (DAPI) Staining. Apoptotic cells were identified on the basis of nuclear morphology changes visualized by DAPI staining. PNT1A and DU 145 cells were harvested, suspended in the growth medium mentioned above, and seeded on 6-well plates (PNT1A were seeded at the density of 1.2×10^5 per well and DU 145 at the density of 1.5×10^5 per well). After 24 h, the cells were washed twice with PBS. Next, the cells were incubated in serum-free media for 24 h in the presence or absence (control) of EPFP (75 μM GAE). The cells were harvested and washed with PBS. Cell fixation was carried out in two stages: first with 1% formaldehyde solution in PBS (pH 7.4) for 15 min in ice and then with ice-cold 70% aqueous solution of ethanol (30 min in ice). The fixed cells were stained with 1 μg/mL DAPI for 30 min in ice at dark. Afterward the stained cells were placed on glass slides, covered with mounting medium, and visualized under a fluorescence microscope (Olympus GX71) at the magnification of 200×.

Statistical Analysis. Data are presented as mean ± SD or SEM, as indicated in figure captions. The number of independent experiments is given in figure captions. Statistical significance of differences between means was determined by nonparametric analysis (Kruskal−Wallis one-way ANOVA followed by Bonferroni test) with the use of Analyze-it software, v. 2.21 (Analyze-it Software, Ltd., Leeds, U.K.); p values of <0.05 were considered to be statistically significant. EQ system with Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA) was used.

Table 1. Primer Sequences for Q-PCR That Were Designed with Primer3 Software[a]

gene	sequence (5′ → 3′)	product length (bp)	T_a (°C)
B2M	TGAGTGCTGTCTCCATGTTTGA	88	55
	TCTGCTCCCCACCTCTAAGTTG		
GAPDH	TCCTTGGAGGCCATGTGGGCCAT	240	55
	TGATGACATCAAGAAGGTGGTGAAG		
MMP1	AGCTCAGGATGACATTGATGG	198	55
	AGCTCAACTTCCGGGTAGAAG		
MMP2	CCGTCGCCCATCATCAAGTT	169	60
	CTGTCTGGGGCAGTCCAAAG		
MMP9	CCTGGAGACCTGAGAACCAATC	104	55
	GATTTCGACTCTCCACGCATC		
MMP14	TCAAGGAGCGCTGGTTCTG	178	55
	AGGGACGCCTCATCAAACAC		
Bcl-2	TTGGCCCCCGTTGCTTTTCCTC	122	56
	TCCCACTCGTAGCCCCTCTGCGAC		
Bax	AGAGGTCTTTTTCCGCGTGGCAGC	137	56
	TTCTGATCAGTTCCGGCACCTTG		
VEGF	AAGGAGGAGGGCAGAATCAT	226	55
	ATCTGCATGGTGATGTTGGA		
c-Fos	CGGGCTTCAACGCAGACTA	147	60
	GGTCCGTGCAGAAGTCCTG		
c-Jun	TGCCTCCAAGTGCCGAAAAA	143	60
	TGACTTTCTGTTTAAGCTGTGCC		

[a]TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany. T_a = annealing temperature.

RESULTS

Characterization of EPFP Composition. The composition of EPFP was determined by both spectrophotometric and HPLC methods (Table 2). A representative chromatogram for the analysis of EPFP components is depicted in Figure 1. According to the colorimetric method with Folin−Ciocalteu reagent phenolics constituted 83% of EPFP. The results of both the colorimetric method with vanillin reagent and HPLC indicated that flavanols were the major phenolic compounds in EPFP because they accounted for approximately 50% of the total polyphenols. Hydrolyzable tannins, especially gallotannins (196.76 ± 13.31 mg/g of EPFP), were the second major group of polyphenols. The content of phenolic acids (ellagic and gallic acid) and flavonols was significantly lower. Comparison of the HPLC retention time and UV spectra maximum with the standards revealed the presence of flavanol monomers, such as (+)-catechin and (−)-epicatechin, as well as the presence of procyanidins: B1, B2, and C1. The total content of the identified oligomers was 250.98 mg/g of EPFP.

Effect of EPFP on Proliferation of PNT1A and DU 145 Cells. As demonstrated in Figure 2, we assessed the influence of the evening primrose flavanol preparation (EPFP), gallic acid (GA), (+)-catechin (CAT), and (−)-epigallocatechin 3-gallate (EGCG) on the proliferation of PNT1A immortalized prostate cells (Figure 2A) and DU 145 prostate cancer cells (Figure 2B). As GA and CAT are EPFP constituents and EGCG is a well-known green tea polyphenol, we included those phenolics in our experimental setup as reference compounds. EPFP tested in our study reduced DU 145 cell proliferation; at the concentration of 100 μM GAE it decreased by 15%, 30%,

Table 2. Characteristics of EPFP[a]

Spectrophotometric Analysis	
total polyphenols[b]	830.09 ± 28.26
total flavanols[c]	422.46 ± 9.11
total proanthocyanidins[d]	201.60 ± 7.82
HPLC Analysis	
total flavanols and HBA[e]	477.88 ± 11.54
(+)-catechin	72.40 ± 3.52
(−)-epicatechin	59.92 ± 2.36
procyanidin B1	76.50 ± 1.18
procyanidin B2	99.30 ± 2.10
procyanidin C1	75.18 ± 2.04
ellagitannins[f]	2.10 ± 0.01
gallotannins[g]	196.76 ± 13.31
free ellagic acid	1.58 ± 0.06
free gallic acid	18.43 ± 0.35
flavonols[h]	1.22 ± 0.09

[a] mg/g dry preparation; mean ± SD, $n \geq 3$. [b]Determined by Folin−Ciocalteu reagent as gallic acid equivalents. [c]Determined by vanillin reagent as (+)-catechin equivalents. [d]Determined by acid hydrolysis as cyanidin equivalents. [e]Determined by HPLC at 280 nm as gallic acid equivalents. HBA = hydroxybenzoic acids. [f]Determined by HPLC at 254 nm by acid hydrolysis as ellagic acid. [g]Determined by HPLC at 280 nm by acid hydrolysis as methyl gallate. [h]Determined by HPLC at 360 nm as quercetin equivalents.

and 51% after 24, 48, and 72 h, respectively. After 72 h incubation with EPFP, the number of live DU 145 cells was reduced in a concentration-dependent manner, and IC_{50} was equal to 97 μM GAE. Interestingly, EPFP stimulated PNT1A cell proliferation at the whole concentration range chosen for the study. The stimulation levels were comparable for all the incubation times; after 72 h it reached 49%, 54%, 37%, and 17% at 25, 50, 75, and 100 μM GAE, respectively. GA and CAT did not influence DU 145 proliferation, contrary to EGCG which reduced the proliferation of the cell line in question (IC_{50} = 65 μM for 72 h). In the case of PNT1A cells, lower concentrations (25−50 μM) of GA and EGCG stimulated cell growth whereas at the concentrations of 75−100 μM cell number reduction was observed. The influence of CAT on PNT1A proliferation was very similar to the effect observed for EPFP. After 72 h CAT stimulated the proliferation of those cells by 40%, 46%, 45%, and 42% at 25, 50, 75, and 100 μM, respectively, with reference to control (untreated cells).

Suppression of Invasiveness of Prostate Cells by EPFP. As demonstrated in Figure 3A, EPFP suppressed the invasiveness of nontumorigenic SV40-immortalized human prostatic epithelial cell line (PNT1A), which are characterized by a low invasiveness, and of highly invasive human tumorigenic cell line (DU 145). For PNT1A the inhibition was pronounced and EPFP concentration-dependent; after 48 h it reached 36%, 48%, and 67% at 25, 50, and 75 μM GAE. In the case of DU 145 the suppression was weaker; however, at the concentrations of 50 and 75 μM GAE it was statistically significant and reached 15% and 24%, respectively. For comparison, we assessed the influence of EGCG, a well-known green tea polyphenol, on cell invasiveness (Figure 3B). EGCG proved to be a very strong inhibitor of PNT1A invasiveness; at 25 μM it decreased by 59% versus control and at 50 μM the reduction reached 98% versus control. At the highest concentration tested in the present work (75 μM) EGCG completely inhibited PNT1A invasiveness (100% inhibition). The suppressive effect of EGCG on DU 145 invasiveness was definitely weaker than in the case of PNT1A and comparable to the effect of EPFP (except for the highest concentration). For instance, 75 μM EGCG inhibited PNT1A invasiveness by 38% versus control. In conclusion, both EPFP (the tested preparation) and EGCG (the reference polyphenol) exhibited a definitely more pronounced ability to suppress invasiveness in the case of PNT1A than for DU 145.

Inhibition of Type IV Collagenase Activity by EPFP. As type IV collagenases (MMP-2 and MMP-9) are able to degrade extracellular matrix (ECM) proteins, which results in destruction of structural barriers during cell invasion (under both physiological and pathological conditions), we decided to assess the influence of EPFP on the activities of those enzymes. The activity of MMP-9 secreted to culture media by PNT1A normal prostate cells and DU 145 prostate cancer cells was very low; hence, we used TNF-α and/or TPA as MMP-9 stimulants in order to investigate the influence of EPFP on the activity of this enzyme. EPFP inhibited the activities of both MMP-2 and MMP-9 secreted by PNT1A in a concentration-dependent manner (Figure 4A). However, MMP-2 inhibition was definitely stronger than in the case of MMP-9 and reached 37%, 66%, 76%, and 84% versus control at 25, 50, 75, and 100 μM GAE, respectively. As shown in Figure 4A, at 75 and 100 μM GAE MMP-2 activity decreased more than 4- and 5-fold, respectively. In the case of MMP-9 EPFP at the concentrations of 25 and 50 μM GAE did not cause any significant changes in

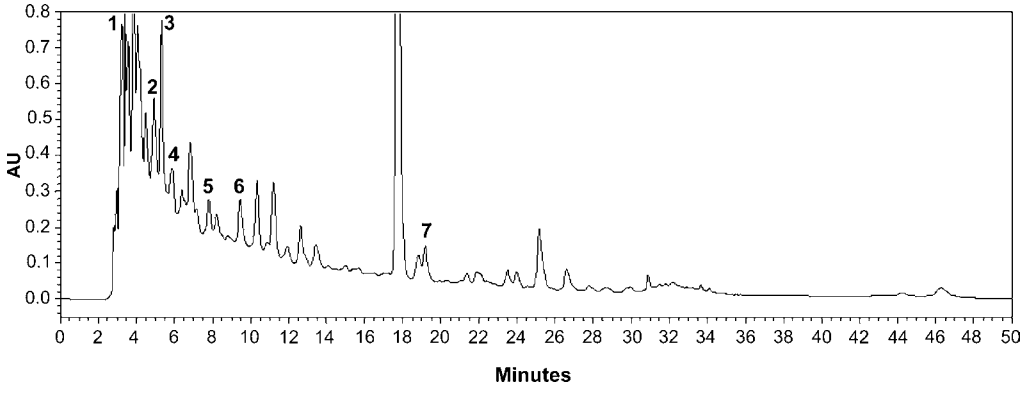

Figure 1. HPLC phenolic profile of EPFP. Detection at 280 nm. Peaks: (1) gallic acid; (2) procyanidin B1 (dimer); (3) procyanidin B2 (dimer); (4) (+)-catechin (procyanidin monomer); (5) procyanidin C1 (trimer); (6) (−)-epicatechin (procyanidin monomer); (7) ellagic acid.

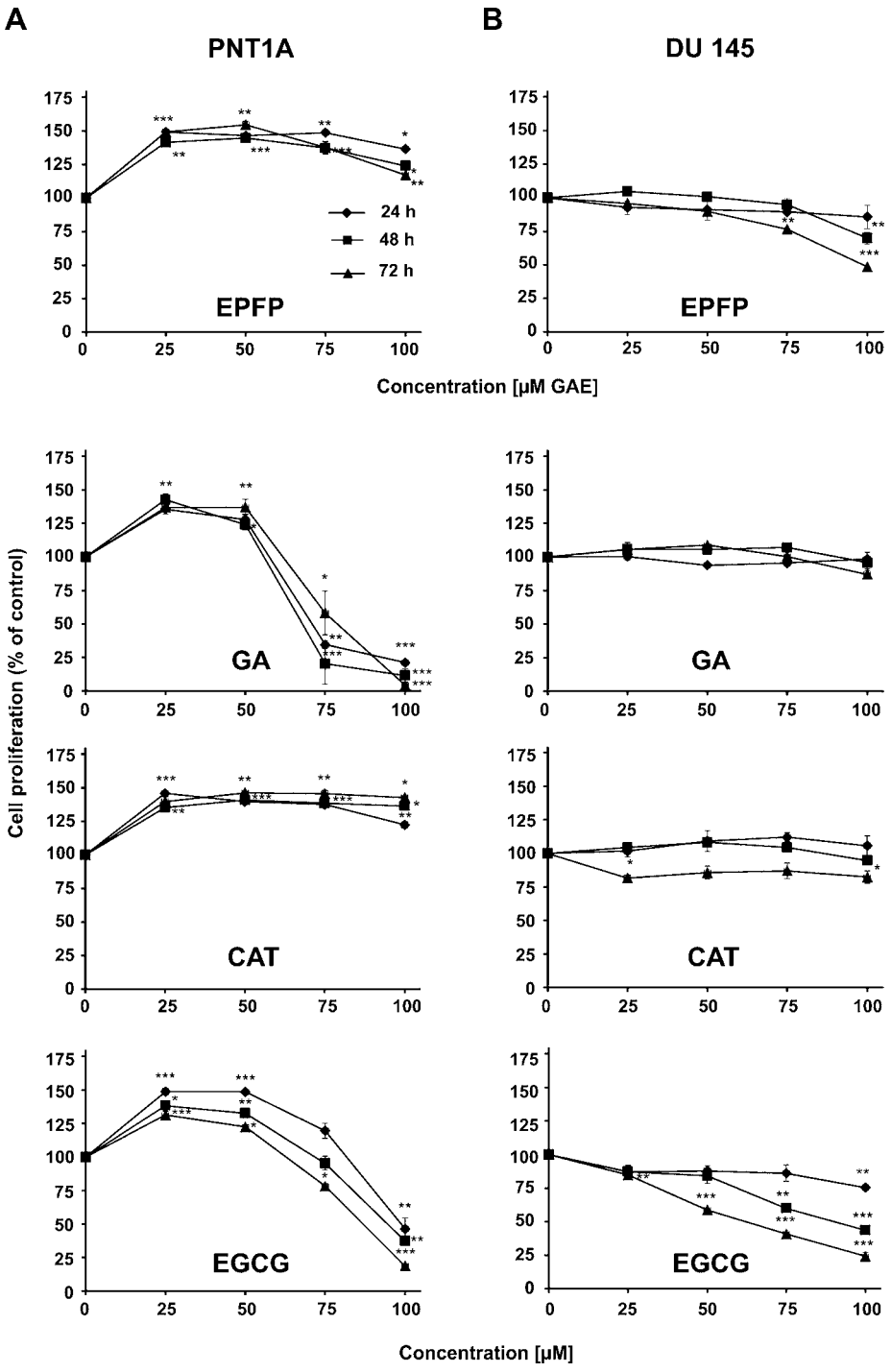

Figure 2. Influence of evening primrose flavanol preparation (EPFP), gallic acid [GA, (+)-catechin (CAT), and (−)-epigallocatechin 3-gallate (EGCG)] on PNT1A (A) and DU 145 (B) cell proliferation determined on the basis of crystal violet staining. Data are expressed as the percentage of control (untreated cells). Each value represents the mean value ± SD, $n = 3$ independent experiments (each experiment was carried out in four replicates). Significance of differences between means: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus control.

the activity of this enzyme. At higher EPFP concentrations, namely, 75 and 100 μM GAE, the inhibition was equal to 27% and 34% versus control, respectively. No MMP-2 activity was detected in DU 145-conditioned medium. EPFP reduced the activity of MMP-9 secreted to culture medium by DU 145 cells in a concentration-dependent manner; the reduction reached 26%, 32%, and 37% at 50, 75, and 100 μM GAE, respectively (Figure 4B). DU 145-derived MMP-9 was inhibited more strongly by EPFP than MMP-9 secreted by PNT1A cells. Interestingly, EGCG totally suppressed (100% inhibition vs control) the activities of both type IV collagenases in PNT1A cells at the whole concentration range chosen for our study (25−100 μM). The inhibition of DU 145-derived MMP-9 by EGCG reached 38% and 51% at 50 and 100 μM, respectively.

Changes in Expression of Selected Genes Involved in Angiogenesis, Metastasis, and Apoptosis upon Incubation with EPFP. In order to partly elucidate the mechanisms of cell proliferation stimulation and inhibition for normal and

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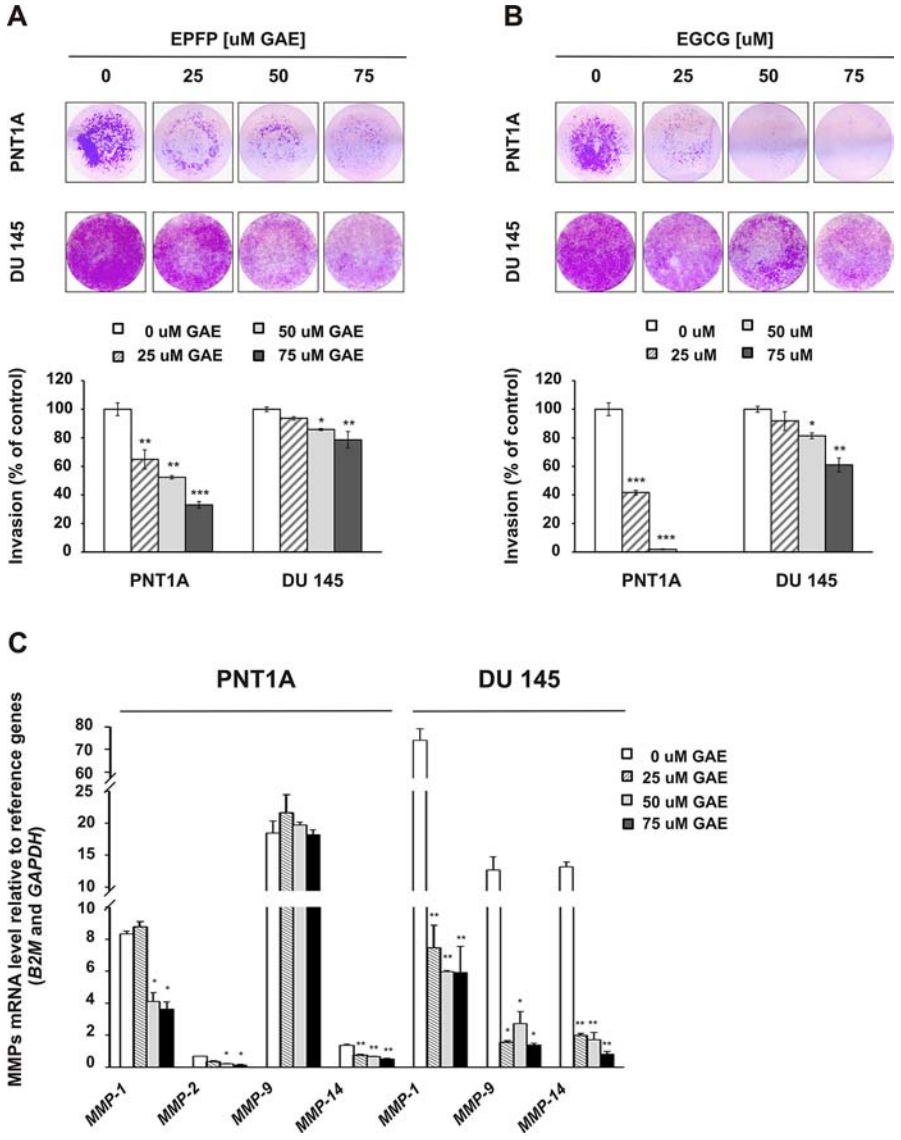

Figure 3. Inhibition of PNT1A and DU 145 cell invasiveness by EPFP (A) and EGCG (B) in the concentration range 0−75 μM GAE/or μM. Invasion studies were conducted using the Matrigel BM matrix assay developed for measurement of tumor cell invasiveness. Total area of inserts with crystal-violet-stained cells was photographed and measured using NIH ImageJ analysis software. Exemplary images of cell growth in Matrigel after 48 h of culture are shown above graphs. Initial magnification ×200. Each value on the graph represents the mean value ± SD, $n = 3$ independent experiments (each experiment was carried out in triplicate). Significance of differences between means: *, $p < 0.05$, **, $p < 0.01$, ***,$p < 0.001$ versus control. (C) Relative *MMPs* (*MMP-1*, *MMP-2*, *MMP-9*, *MMP-14*) mRNA expression levels measured by real-time quantitative RT-PCR analysis in two cell lines: PNT1A and DU 145. Each value on the graphs represents the mean value ± SD, $n = 3$ independent experiments (each experiment was carried out in triplicate): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

cancer prostate cells, respectively, and the mechanisms of invasiveness suppression for both types of cells, we determined the influence of the studied preparation on expression levels of selected genes involved in degradation of extracellular matrix, angiogenesis, and apoptosis. As presented in Figure 5, after 16 h of incubation EPFP differently modulated gene expression versus control (untreated cells) in the cell lines chosen for our study. In PNT1A EPFP caused a concentration-dependent down-regulation of *MMP-1*, *MMP-2*, and *MMP-14*. At 75 μM GAE the expression levels of *MMP-1*, *MMP-2*, and *MMP-14* were equal to 0.43-, 0.36-, and 0.38-fold control, respectively. In normal prostate cells only *MMP-9* expression level remained unchanged (no statistically significant differences were observed) after incubation with EPFP at the whole concentration range. On the contrary, for DU 145 cells the decrease in *MMP-9* expression was very pronounced (0.10-fold control). The decrease in the expression levels of *MMP-1* (0.08-fold control) and *MMP-14* (0.06-fold control) at 75 μM GAE in prostate cancer cells was also much more pronounced than in the case of normal prostate cells. In both cell lines incubation with EPFP resulted in down-regulation of *Bcl-2* and *Bax* (antiapoptotic and pro-apoptotic effector molecules, respectively), albeit to different extents. *Bcl-2* expression was suppressed to a much higher extent than in the case of *Bax*. In DU 145 *Bcl-2* down-regulation was strong and concentration-dependent; for instance, at 50 μM GAE its expression level was 0.02-fold control. On the other hand, in PNT1A cells EPFP tested at the same concentration *Bcl-2* expression level was equal to 0.40-fold control. Taking into account the changes in expression levels of genes involved in apoptosis in PNT1A

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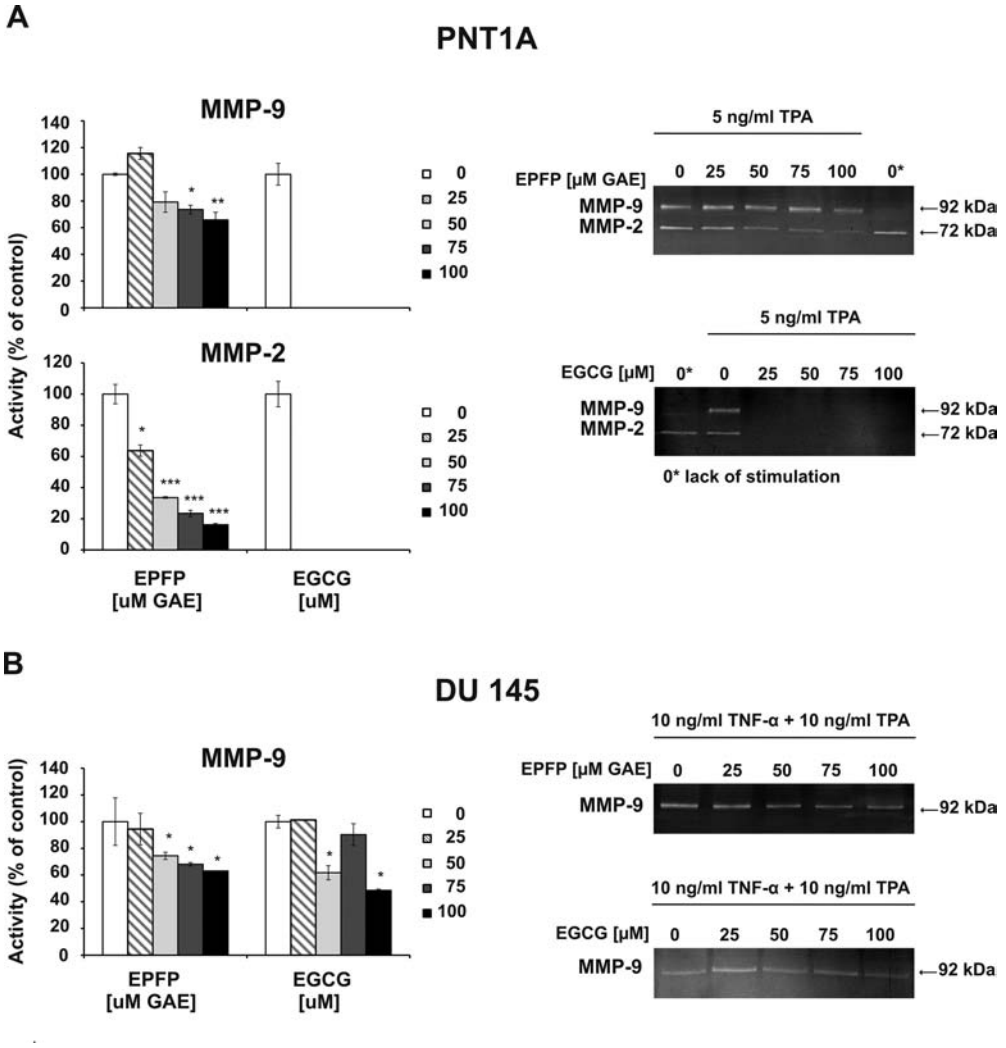

Figure 4. Inhibition of type IV collagenase (MMP-2 and MMP-9) activities after incubation of PNT1A (A) and DU145 (B) cells with EPFP. Zymographic analysis of the media was carried out after 48 h incubation with EPFP or with EGCG (reference polyphenol). Representative zymograms obtained after 48 h incubation with EPFP or EGCG are shown next to graphs. Each value on the graph represents the mean value ± SEM, $n \geq 3$ independent experiments. Significance of differences between means: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control.

and DU 145 cells resulting from incubation of the cells with EPFP, we decided to present *Bcl-2* mRNA to *Bax* mRNA ratio (Figure 6A). In normal prostate cells the *Bcl-2/Bax* ratio increased, while in prostate cancer cells the ratio decreased in EPFE concentration-dependent manner. The studied flavanol preparation caused a reduction in vascular endothelial growth factor (*VEGF*) expression level in both cell lines; however, the down-regulation was more pronounced in the case of cancer cells. We also determined the influence of EPFP on the expression of two transcription factors, namely, *c-Fos* and *c-Jun*. As demonstrated in Figure 5, suppression of both factors was very strong in cancer cell line. At the lowest concentration tested (25 μM GAE) *c-Fos* and *c-Jun* expression levels were 0.17- and 0.25-fold control, respectively. It is worth emphasizing that EPFP did not cause any significant changes in *c-Jun* expression in PNT1A cells; at 25 μM GAE the expression level of this transcription factor was even higher than in untreated cells (1.3-fold control). In order to elucidate the role of particular MMPs in cell invasiveness, we presented the relative mRNA expression levels of those enzymes in Figure 3C. Taking into account the expression levels in controls (untreated cells), we observed that for PNT1A cells the level of *MMP-9* was the highest, while the levels of *MMP-1*, *MMP-2*, and *MMP-14* were 2.2-fold, 27.5-fold, and 13.6-fold lower, respectively (in comparison with *MMP-9*). In the case of DU 145 the highest expression level was observed for *MMP-1* whereas the expression levels of both remaining MMPs (*MMP-9* and *MMP-14*) were comparable and 5.7-fold lower than *MMP-1* expression level.

Detection of Apoptosis by DAPI Staining. After 24 h incubation of normal prostate PNT1A cells and prostate cancer DU 145 cell line with EPFP at 75 μM GAE we observed morphology of cell nuclei stained with DAPI (Figure 6B). Contrary to normal cells, DU 145 cell number was reduced and their nuclei exhibited changes specific to apoptosis (chromatin condensation, nuclear shrinkage). For all the three tested agents (namely, EPFP, EGCG, and camptothecin) nuclear DNA fragmentation typical for late stages of apoptosis was observed in DU 145 cancer cells.

DISCUSSION

In recent years much attention is paid to plant-derived phenolics. Those compounds exhibit pleiotropic biological activities; among others, antioxidant, anti-inflammatory, and

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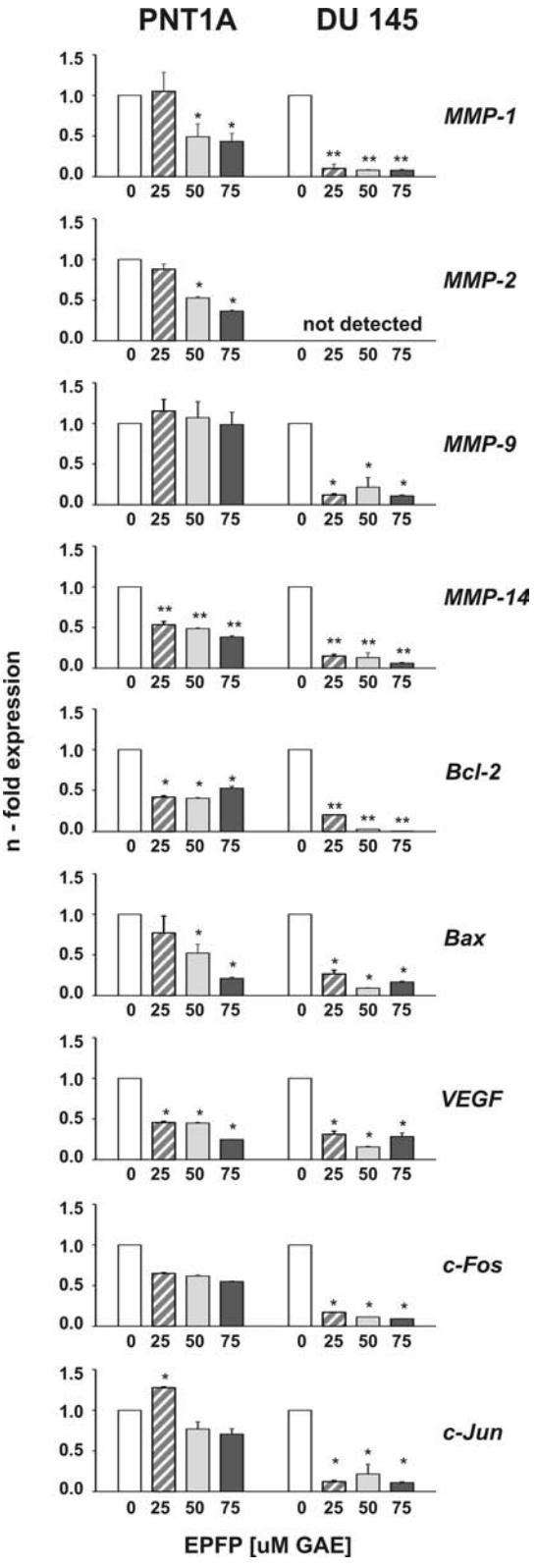

Figure 5. Quantitative real time RT-PCR analysis of selected genes in PNT1A and DU 145 cells incubated for 16 h with EPFP at the concentrations of 25, 50, and 75 μM GAE. Control expression level obtained for untreated cells was taken as 1. Each value on the graphs represents the mean value ± SD, $n = 3$ independent experiments (each experiment was carried out in triplicate). Significance of differences between means: *, $p < 0.05$; **, $p < 0.01$ versus control.

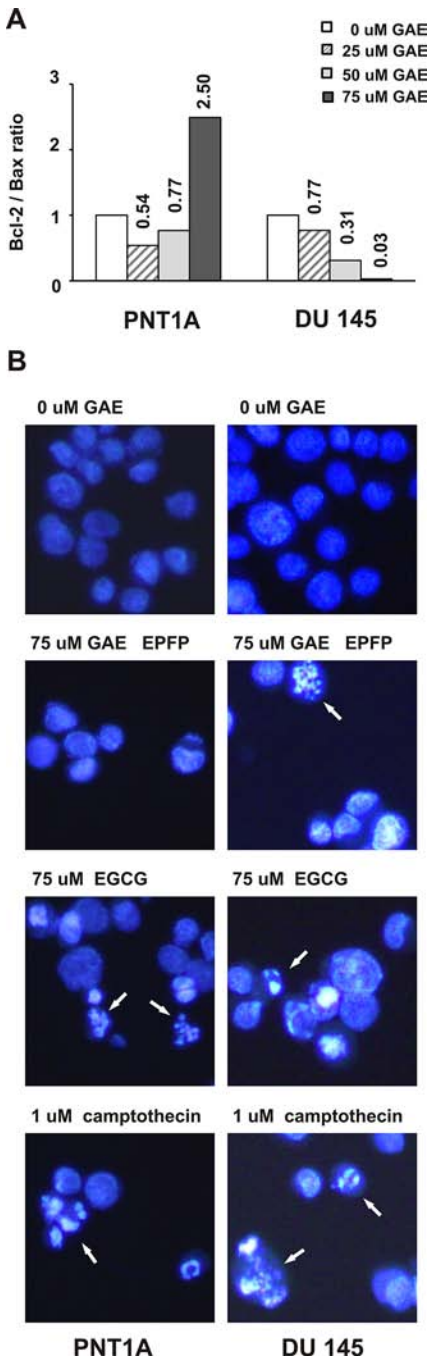

Figure 6. (A) Changes in the ratio of *Bcl-2* and *Bax* mRNA expression levels in PNT1A and DU 145 cells. (B) The ability of EPFP to induce apoptosis in PNT1A and DU 145 cells after 24 h incubation at the concentration of 75 μM GAE. Morphological changes in cell nuclei specific to apoptosis were detected after DAPI staining and are indicated with arrows. Initial magnification: 200×, UV filter.

anticancer. In the present work we investigated a flavanol preparation (EPFP) obtained from defatted seeds of evening primrose (*Oenothera paradoxa*) as a putative anticancer agent. Our experimental setup comprised normal immortalized prostate cells (PNT1A) and prostate cancer cells (DU 145). We determined the influence of EPFP (rich in procyanidins and their monomers, (+)-catechin and (−)-epicatechin on proliferation of both cell lines (Figure 2). EPFP suppressed cancer cell growth (IC_{50} = 97 μM GAE after 72 h incubation), contrary to two reference compounds (GA and CAT) which

were both identified as its constituents (Table 2). The advantage of extracts over their isolated components results from the fact that the bioactivities of various extract constituents complete one another and from the synergistic interactions among them.[29,30] Therefore, on the basis of the obtained results one might suggest that the antiproliferative activity of EPFP could result from synergistic interactions among its components. Not only EPFP but also another reference compound (EGCG) inhibited DU 145 cell growth. Interestingly, EPFP did not reduce PNT1A proliferation; in contrast, a stimulation of cell growth was observed (by 49% at 25 μM GAE after 72 h incubation). A similar effect was noticed for CAT. Both EGCG and GA at lower concentrations (25 and 50 μM) stimulated PNT1A proliferation whereas at higher concentrations (75 and 100 μM) cell growth inhibition was observed. Those results could be explained as a hormetic effect which has very important implications for, among others, antibacterial, antiviral, and anticancer therapies.[31]

A hormetic effect was observed for garcinol, a phenolic compound isolated from the skin of Indian mangosteen fruit. At higher concentrations (>1 μM) garcinol inhibited proliferation of two colon cancer cell lines (HT-29 and HCT-116) and normal intestinal epithelial cells (IEC-6), whereas at lower concentrations (<1 μM) proliferation of those cell lines was stimulated.[32] Stimulation and inhibition, respectively, of PNT1A and DU 145 cell growth by EPFP could be explained by the fact that the preparation differently modulated *Bcl-2/Bax* ratio in those cells. As shown in Figure 6A, the *Bcl-2/Bax* ratio in PNT1A cells increased and in DU 145 it decreased at the whole concentration range chosen for our study.

We did not find any report on the influence CAT on the growth of PNT1A and DU 145 cells. However, there are works regarding the influence of GA and EGCG on the growth of DU 145 cell line. Growth assays, incubation times, and concentration ranges of GA chosen by authors of those papers are different from those used in our experimental setup. In one of the reports a grape seed extract was tested as a potential anticancer agent, and the authors concluded that GA (25−50 μg/mL, which is equivalent to 147−294 μM) was the major active compound responsible for the observed inhibition of DU 145 cell growth.[33] After 24 h incubation DU 145 growth was inhibited by 55% and 60% at 147 and 294 μM, respectively. For comparison, the concentration range chosen for our studies was from 25 to 100 μM; at such concentrations we did not observe any statistically significant influence of GA on the growth of DU 145 cell line. In the next two papers MTT[34] and XTT[35] assays were employed while we used crystal violet staining. Thus, the differences in the obtained results may be caused by the use of different assays. Russell et al.[36] assessed the influence of GA on cancer cell growth on the basis of the same technique as in our experimental setup; however, the cell lines used in that study were different, namely, prostate cancer cells (LNCaP) and prostate normal epithelial cells (PrEC). In the case of LNCaP after 72 h incubation with GA IC_{50} was equal to 48 μg/mL (282 μM). Thus, the concentrations of this phenolic acid were several times higher than those used in our studies. After 24 h GA did not cause any significant changes in PrEC cell growth. As far as the influence of EGCG on DU 145 cells is concerned, the results of our investigatons are similar to those presented in the paper by Ravindranath et al.[37] where IC_{50} = 89 μM. In our studies after 72 h incubation of DU 145 cells with GA IC_{50} was equal to 65 μM.

Our next experiment proved that EPFP strongly inhibited PNT1A invasiveness (67% vs control at 75 μM GAE); suppression of invasiveness was less pronounced in the case of DU 145 cells (24% vs control at 75 μM GAE) (Figure 3A). Similar difference in reducing invasiveness of those cell lines was observed for EGCG (Figure 3B). Such results indicate that DU 145 cells are more resistant to the anti-invasive activity of the evening primrose flavanols than PNT1A cells. In order to explain the above-mentioned differences we determined the influence of EPFP on transcription of *MMP-1*, *MMP-2*, *MMP-9*, and *MMP-14*. Comparing differences in the changes in expression levels of particular *MMPs* (Figures 3C and 5) and differences in the suppression of invasiveness (Figure 3A) can lead to the conclusion that MMP-9 does not play a crucial role in this process. In PNT1A cells, for which a strong decrease in invasiveness was observed, *MMP-9* expression was even slightly stimulated by EPFP. In contrast, in DU 145 cell line the reduction of invasiveness was not pronounced and *MMP-9* was very strongly down-regulated (0.1-fold control at 75 μM GAE). Therefore one may conclude that EPFP suppressed PNT1A invasiveness through lowering the transcription (and, consequently, activity) of *MMP-1*, *MMP-2*, and *MMP-14*. In DU 145 cells the decrease in invasiveness could result mainly from lowering the transcription of *MMP-1* and *MMP-14*. The results mentioned above are in line with the fact that EPFP also inhibited the transcription of *VEGF* in both cell lines, which could imply an antiangiogenic activity of the investigated preparation. VEGF is an angiogenesis stimulator; it causes an increase in MMP-2 synthesis and indirectly participates in its activation.[38] It is worth emphasizing that EPFP down-regulated *MMP-14*, a membrane-type metalloproteinase which plays a key role in the activation of MMP-2 which in turn is essential for angiogenesis.[39] On the other hand, a decrease in *VEGF* expression in normal cells may inhibit physiological angiogenesis occurring in such processes as wound healing.[40]

Furthermore, in cancer cells EPFP strongly down-regulated two transciption factors, *c-Jun* and *c-Fos*, whereas in normal cells it stimulated *c-Jun* expression at 25 μM GAE, which could explain the slight stimulation of *MMP-1* and *MMP-9* expression observed at 25 μM GAE. As promoter regions of *MMP-1* and *MMP-9* genes comprise c-Fos- and c-Jun (*activator protein 1*/AP-1)-binding sites,[41,42] it seems probable that EPFP could modulate the expression levels of those *MMPs* in prostate cells by inducing changes in the transcription of *c-Fos* and *c-Jun*. Among polyphenols, EGCG, resveratrol, and curcumin (among others) were identified as agents inhibiting AP-1 activation.[43,44]

As type IV collagenases (MMP-2 and MMP-9) can destroy structural barriers by degrading extracellular matrix components, and thus render possible cell invasion (both physiological and pathological),[45,46] we assessed the influence of EPFP on their activities. In PNT1A cells MMP-2 inhibition by EPFP occurred not only at the level of transcription, but also at the protein level, which is in accordance with our hypothesis concerning suppression of PNT1A invasiveness by EPFP through this enzyme. In the case of MMP-9 transcription was not reduced; hence, probably, inhibition of the enzyme activity resulted from a decrease in its secretion and/or from binding of EPFP constituents directly to the enzyme, or from cross-linking its substrate and thus protecting it against proteolysis.[47] In DU 145 cell line EPFP inhibited MMP-9 activity on both mRNA and protein levels. There is literature data on inhibition of aminopeptidase N (APN) and neutral endopeptidase (NEP) activities by a polyphenol-rich extract from evening primrose

seeds.[48] This result is in line with the results of our study, since APN (similarly to MMP-2 and MMP-9) participates in invasion, metastasis, and angiogenesis.[49] Besides, the inhibition of the activity of MMP-9 secreted to culture medium by DU 145 cells by EGCG (the reference polyphenol in our experimental setup) is in accordance with the results reported by Vayalil et al.[50]

Apoptosis induction is looked upon as an effective therapeutic strategy[51] and as a promising chemopreventive approach.[52] The number of reports on the proapoptotic activity of various phenolics toward prostate cancer cells is increasing.[53,54] We decided to present the ratio of *Bcl-2* expression level to *Bax* expression level in this work (Figure 6A), because EPFP tested in our study down-regulated *Bcl-2* more strongly than *Bax* in DU 145 cells (Figure 5). For DU 145 cells the ratio decreased from 1.0 to 0.03 within the concentration range used in our study. If the *Bcl-2/Bax* ratio is lower than 1, the cells are sensitive to proapoptotic agents,[55] as evidenced in the study by Hastak et al.[53] where EGCG induced apoptosis of prostate cancer LNCaP cells by modulating Bax and Bcl-2 levels. An additional confirmation of the proapoptotic activity of EPFP toward DU 145 cells is provided by the results presented in Figure 6B where characteristic apoptotic bodies are visible. Our results for normal prostate PNT1A cells demonstrate that *Bcl-2/Bax* ratio increases in a concentration-dependent manner, thus rendering the cells more resistant to apoptosis inducing agents. On the other hand, in the case of normal prostate cells the increase in the above-mentioned ratio may result in cell growth and undesirable changes in the form of benign prostate hyperplasia (BPH).[56,57] The proapoptotic activity of a flavanol preparation from evening primrose seeds was demonstrated previously toward colon cancer Caco-2 cells.[9] Apoptosis induction in cancer cells by EPFP may result from the presence of, among others, ellagic acid in this preparation (Table 2). This compound was identified in an extract from evening primrose seeds in the free form and as a structural element of an ellagitannin, oenothein B.[48] The acid was proved to trigger apoptosis in colon cancer Caco-2 cells, breast cancer MCF-7 and Hs 578T cells, and prostate cancer DU 145 cells, and did not exhibit antiproliferative activity toward normal fibroblasts at the concentration range from 10 to 100 μM.[58] Apoptosis induction in Caco-2 cell line upon incubation with ellagic acid was also observed by Larrosa et al.;[59] importantly, normal colon epithelial cells (CCD-112CoN) were resistant to the proapoptotic activity of this compound.

In summary, our work demonstrates for the first time that EPFP reduces prostate cancer DU 145 cell proliferation and suppresses their invasiveness. EPFP did not cause a reduction in the number of normal prostate cells; it effectively inhibited PNT1A invasiveness. Furthermore, EPFP also caused favorable changes in Bcl-2/Bax mRNA ratio which render cancer prostate cells (DU 145) more sensitive to apoptosis-triggering agents. Our results indicate that EPFP (a waste product of pharmaceutical and cosmetic industries) could be used as a source of prostate cancer cell apoptosis inducers, as well as inhibitors of MMPs and VEGF which are involved in angiogenesis and metastasis. Therefore, in vivo studies on the chemopreventive and therapeutic activities of the evening primrose flavanols seem to be warranted.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

EPFP, evening primrose flavanol preparation; GAE, gallic acid equivalent; EGCG, (−)-epigallocatechin 3-gallate; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; TNF-α, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VEGF, vascular endothelial growth factor

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